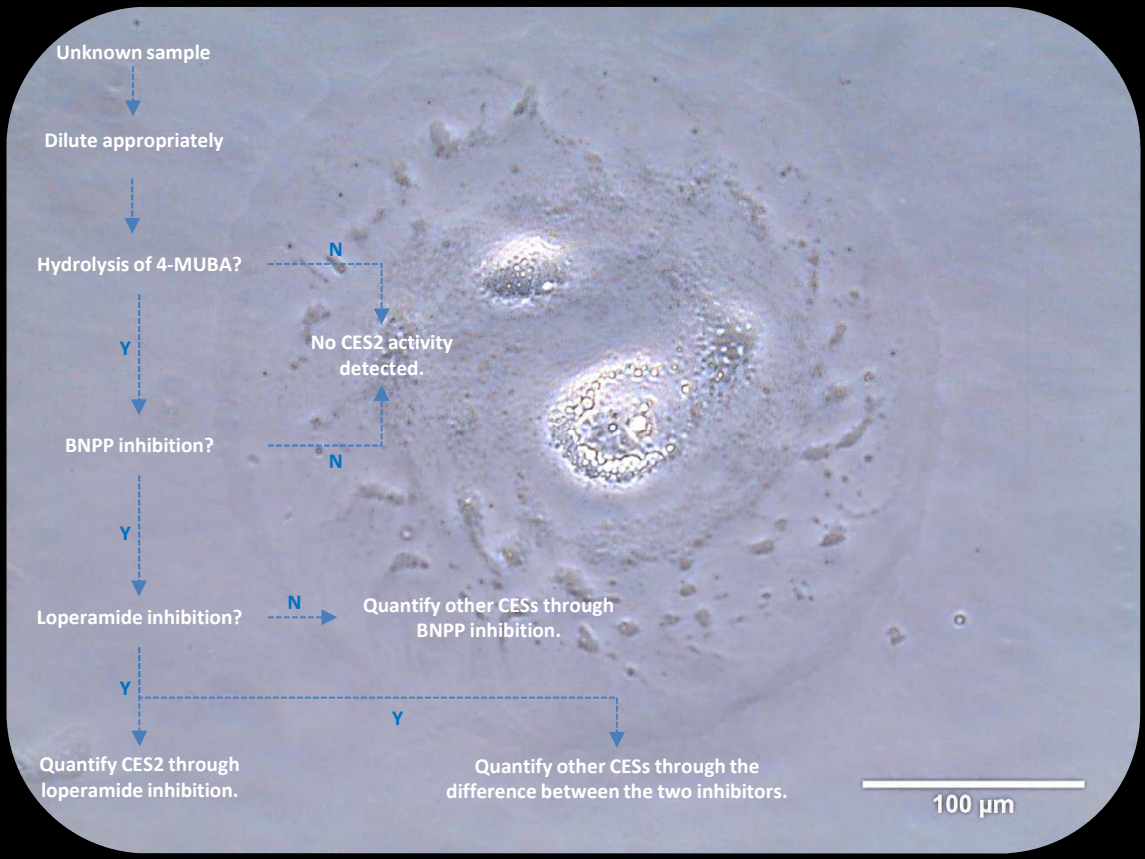
Understanding Human Carboxylesterase 2

And the feasibility of a more relevant cell model for intestinal metabolism

Joana Lamego



Dissertation presented to obtain the Ph.D. degree in Engineering and Technology Sciences, Biotechnology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras, December, 2012

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA /UNL
Knowledge Creation

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ITQB-UNL and IBET, Pharmacokinetics and Biopharmaceutical Analysis Laboratory and Cell Line Development and Molecular Biotechnology Laboratory - Animal Cell Technology Unit
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Foreword

This thesis dissertation is the result of four years of research at the Pharmacokinetics and Biopharmaceutical Analysis Laboratory and at the Cell Line Development and Molecular Biotechnology Laboratory - Animal Cell Technology Unit of *Instituto de Tecnologia Química e Biológica – Universidade Nova de Lisboa* and *Instituto de Biologia Experimental e Tecnológica* (Oeiras, Portugal) under the supervision of Dr. Ana Luísa Simplício and co-supervision of Dr. Ana Sofia Coroadinha.

The work presented herein intended to contribute to the improvement of Carboxylesterases (CESs) knowledge through the development of new analytical methodologies as well as new production processes enabling enzymatic analysis and characterisation. Moreover, this thesis also contributes with an improved *in vitro* Caco-2 cell model showing higher levels of human carboxylesterase 2 (hCES2) expression and activity.

Aos meus pais

Aos que me ajuda(ra)m a ser

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conhecimento, inovação, empreendedorismo e excelência em biotecnologia; agradeço ainda todo o apoio que me deu e, em especial, às vezes que me "espicaçou" cientificamente para ser e fazer mais e melhor, contribuindo de uma forma decisiva para o meu crescimento a nível científico e pessoal.

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Um obrigada muito especial à Bárbara Cunha, co-autora desse mesmo trabalho e companheira de experiências "malucas" (e não tão "malucas" assim) que nunca vira a cara a um bom desafio, a uma boa aventura, mesmo que isso implique trabalhar desenfreadamente até à exaustão. Obrigada pela tua fundamental ajuda, foi um verdadeiro privilégio trabalhar ao teu lado. Serás uma brilhante cientista e podes contar com esta tua amiga para o que precisares.

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iBET FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR
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Abstract

The first barrier oral drugs and prodrugs encounter prior to reaching an organism's systemic circulation is the gastrointestinal (GI) tract, specifically the intestine, which is the primary section for absorption. Therefore, it is fundamental to understand the permeability of the therapeutic agent as well as its potential metabolism by human enterocytes, since biotransformation may result in the inactivation of the therapeutic agent or, to the contrary, in the formation of more therapeutically active metabolites. Carboxylesterases (CESs), phase I metabolising enzymes, are important in the metabolism of several drugs and prodrugs with amide, ester, or thioester functional groups. After cytochrome (CYP) P450s and UDP-glucuronosyltransferases (UGTs), CESs are the most relevant enzymes for the metabolism of therapeutic agents.

Carboxylesterase 2 (hCES2) is the main CES expressed in the human intestine and is an increasingly important enzyme in anti-cancer combined therapies for the treatment of different pathologies such as colon adenocarcinoma and malignant glioma, due to its potential to improve the anti-cancer effect of certain therapeutic compounds. Notwithstanding, it is down regulated in the most used intestinal model for permeability, the human colon adenocarcinoma derived Caco-2 cell line. On the contrary, carboxylesterase 1 (hCES1), the main CES expressed in the human liver, is highly expressed in Caco-2 cells, unlike what is known to occur in the intestine.

The main goal of the work presented in this thesis was to increase the fundamental knowledge of hCES2 and to improve the *in vitro* to *in vivo* relevance of Caco-2 cells, by increasing the expressed hCES2 levels.

To study and characterize hCES2 activity, as well as to differentiate its activity from other esterases in complex biological samples, proper tools had to be developed, as described in Section 2. Through the usage of both a specific CES2 and a general CES inhibitor, loperamide and BNPP, respectively, a fast, simple, and repeatable capillary electrophoresis method requiring low sample volumes was successfully developed

and is applicable even when substrates and products absorb at the same wavelength. The developed methodology was applied to a variety of samples and proved to be suitable for the quantitation of hCES2-specific activity in mixtures of expressed and purified CES1 and hCES2 enzymes. Moreover, the developed method showed that transiently transfected HEK-293T cells had 15-fold higher hCES2-specific activity than non-transfected cells. This method also showed effectiveness in distinguishing CES2-specific activity in different mammalian sera containing a variety of esterases. The developed methodology should be applicable to a wider variety of samples with esterase activity, as well as for the analysis of the activities of other esterases, once appropriate substrates and inhibitors are used.

Using the appropriate tools to study hCES2, a method enabling the production and purification of this enzyme was developed. This successfully strategy, described in Section 3, resulted in the first recombinant human hCES2 enzyme produced using human HEK-293T cells in suspension. The utilized strategy demonstrated that the addition of an in-frame, C-terminally localized 10x histidine tag, was sufficient to promote hCES2 secretion. This avoided the need of additional N-terminal signal sequences or the modification or deletion of the Endoplasmic Reticulum (ER) retention sequence, which are the traditional strategies. Moreover, using both standard and in-house developed biochemical and analytical techniques, new fundamental features were unravelled, such as the presence of oligomeric active and inactive forms of hCES2, which was previously reported as existing only in a monomeric 60 kDa form, leading the way to possible new discoveries concerning hCES2 properties. Different oligomeric forms had previously been reported for hCES1; the present work suggests this might be a common feature for CESs.

Equipped with a deeper knowledge about hCES2, Caco-2 cells were overexpressed with hCES2. This cell line, mimicking human enterocytes, fails in the accurate absorption prediction of ester-containing drugs and prodrugs especially of those

metabolised by hCES2. Section 4 describes the successful genetic engineering of this cell line resulting in a population with increased hCES2 mRNA, protein expression, and activity levels. No changes in cell differentiation and polarization ability were detected in comparison with the parental cell line, as the overexpressing population retained the capacity to express similar levels of alkaline phosphatase. A hCES2-expressing Caco-2 cell line was generated through clonal selection and its stability during passaging and differentiation, was evaluated. An obvious decay of enzyme expression was observed at higher passage numbers, confirming a previously reported hurdle to Caco-2 manipulation. New clues to understand and overcome this limitation of the Caco-2 model are provided. Upon complete validation with reference compounds, the newly developed cell line has the potential to become a useful tool for coupling the study of intestinal absorption with intestinal metabolism, especially when it involves the hydrolysis of ester-containing drugs and prodrugs.

Overall, the work developed in this thesis, opened new paths to unravel more mysteries of hCES2 and Caco-2 cells.

Resumo

Fármacos e pró-fármacos administrados por via oral encontram no trato gastrointestinal, em especial no intestino delgado, região primordial de absorção, a primeira barreira antes de alcançarem o sistema circulatório. Torna-se, desta forma, fundamental, compreender as propriedades dos agentes terapêuticos em termos de permeabilidade, bem como a possível susceptibilidade a serem metabolizados nos enterócitos humanos, uma vez que a sua bio-transformação pode conduzir à inativação, ou, pelo contrário, à formação de metabolitos de maior ação terapêutica. Carboxilesterases (CESs) são enzimas metabólicas de fase I importantes no metabolismo de fármacos e pró-fármacos contendo grupos químicos funcionais específicos tais como amidas, ésteres ou tio-ésteres. Carboxilesterases são, depois das citocromo (CYP) P450 e das UDP-glucoronosil-transferases (UGTs), as enzimas de maior relevância para o metabolismo de agentes terapêuticos.

A carboxilesterase 2 (hCES2) é a principal CES expressa no intestino humano, sendo uma enzima de importância crescente em terapias anti-cancerígenas combinadas para o tratamento de diversas patologias tais como o adenocarcinoma do colon ou o glioma maligno, devido ao potencial que tem de aumentar o efeito anti-cancerígeno de certos compostos terapêuticos. Esta enzima encontra-se, contudo, regulada negativamente no modelo intestinal mais utilizado para a avaliação da permeabilidade intestinal, a linha celular Caco-2, derivada de um adenocarcinoma de colon humano. Pelo contrário, a carboxilesterase 1 (hCES1), a CES de maior expressão no fígado humano, é altamente expressa nas células Caco-2, de modo oposto ao que se sabe ocorrer no intestino humano.

O principal objetivo do trabalho apresentado nesta tese foi o de aumentar o conhecimento fundamental da hCES2 bem como o de melhorar a relevância *in vitro-in vivo* das células Caco-2, através do aumento da expressão dos níveis de hCES2. As ferramentas apropriadas para o estudo e caracterização da atividade da hCES2, bem

como para a diferenciação da atividade desta de outras esterases presentes em amostras biológicas complexas, tiveram de ser desenvolvidas, tal como descrito na Secção 2. A utilização de um inibidor específico da CES2, a loperamida, e de um inibidor geral das CESs, o BNPP, possibilitou o desenvolvimento com sucesso de um método rápido, simples, repetível e necessitando de pequenas quantidades de amostra, para análise em eletroforese capilar, com aplicabilidade mesmo em situações nas quais substratos e produtos de reação absorvem no mesmo comprimento de onda. A metodologia assim desenvolvida foi aplicada em diversos tipos de amostras e provou ser adequada para a quantificação da atividade específica da hCES2, mesmo quando presente em misturas de enzimas expressas e purificadas, contendo CES1. Adicionalmente, o método desenvolvido possibilitou demonstrar que a atividade específica de hCES2 aumenta 15 vezes em células HEK-293T transfectadas de forma transiente em comparação com as mesmas células não transfectadas. Este método provou ainda ser eficaz para distinguir a atividade específica de CES2 presente em diferentes soros de mamíferos, contendo diversas esterases. A metodologia desenvolvida poderá ser adicionalmente aplicada não só a uma maior variedade de amostras contendo atividade de esterases, bem como para a avaliação de atividades de outras esterases desde que sejam utilizados substratos e inibidores apropriados.

Recorrendo às ferramentas apropriadas ao estudo da hCES2, foi desenvolvido um método de produção e purificação desta enzima. Esta estratégia, descrita na Secção 3, revelou-se bem-sucedida, tendo resultado na produção pela primeira vez de uma enzima hCES2 recombinante humana, em células de origem humana, a linha celular HEK-293T, crescidas em suspensão. A estratégia utilizada permitiu demonstrar que a adição, na mesma grelha de leitura, de uma cauda de 10x histidinas na região C-terminal da hCES2, é suficiente para promover a secreção da proteína. Esta abordagem permitiu evitar recorrer às estratégias tradicionais tais como: adição de sequências sinal na região N-terminal; modificação ou delecção da sequência de

retenção no Retículo Endoplasmático (ER). Adicionalmente, através da utilização de técnicas analíticas e bioquímicas quer tradicionais quer desenvolvidas no laboratório, foi possível revelar novas propriedades fundamentais da hCES2 tais como a presença de formas oligoméricas ativas e inativas, desta proteína anteriormente descrita como existindo apenas na forma de monómero com 60 kDa, abrindo caminho a eventuais novas descobertas relativamente às suas propriedades. De facto, diferentes formas oligoméricas foram já descritas para a hCES1; o trabalho aqui apresentado sugere que esta possa ser uma característica comum a todas as CESs.

Munidos de um conhecimento mais aprofundado sobre a hCES2, procedeu-se ao aumento de expressão desta enzima nas células Caco-2. Esta linha celular, que mimetiza os enterócitos humanos, apresenta limitações na previsão da absorção de fármacos e pró-fármacos contendo grupos éster, especialmente daqueles que são metabolizados pela hCES2. Na Secção 4 é descrita a modificação bem-sucedida desta linha celular, através de técnicas de engenharia genética, resultando no estabelecimento de uma população com níveis superiores de atividade de hCES2, reflectida também ao nível do mRNA e ao nível da expressão proteica. Comparando a linha celular Caco-2 com a nova população celular, não foram detectadas diferenças em termos da capacidade de diferenciação celular nem ao nível da capacidade de polarização das células, uma vez que na nova população, com níveis superiores de expressão de hCES2, foram detectados níveis semelhantes de expressão de fosfatase alcalina. Foi observado um decaimento óbvio na expressão enzimática de hCES2, a passagens mais elevadas, confirmando a dificuldade previamente reportada ao nível da manipulação da linha celular Caco-2. Neste trabalho são fornecidas novas indicações de como ultrapassar estas limitações. A nova linha celular desenvolvida tem o potencial de se tornar uma ferramenta importante na conjugação dos estudos de absorção e de metabolismo intestinais, especialmente úteis aquando da presença de hidrólise de fármacos e pró-fármacos contendo grupos éster.

De uma forma global, o trabalho desenvolvido nesta tese permitiu abrir novos caminhos na descoberta de mistérios adicionais da hCES2 e das células Caco-2.

** Este texto foi escrito ao abrigo do novo acordo ortográfico. Nas palavras com grafias alternativas, a nova grafia foi preterida.*

Thesis Publications

Peer-reviewed Articles

Lamego J, Coroadinha AS, Simplício AL. Detection and quantification of carboxylesterase 2 activity by capillary electrophoresis. *Analytical Chemistry* 2011; 83: 881-887.

Lamego J, Cunha B, Peixoto C, Sousa MF, Alves PM, Simplício AL, Coroadinha AS. Carboxylesterase 2 production and characterization in human cells: new insights into enzyme oligomerization and activity. *Applied Microbiology and Biotechnology* 2012; doi 10.1007/s00253-012-3994-3.

Lamego J, Simplício AL, Coroadinha AS. Development of a Caco-2 cell line expressing human Carboxylesterase 2. *Toxicology in vitro* 2012; submitted.

Book Chapters

Simplício AL, Coroadinha AS, Gilmer JF, **Lamego J.** A methodology for detection and quantification of esterase activity. *Methods in Molecular Biology: Electrophoresis of Biomolecules* 2013; in press.

Published Abstracts and Proceedings

Lamego J, Coroadinha AS, Simplício AL. Evaluation of carboxylesterases activity in biological samples. *Revista Portuguesa de Farmácia* 2010; Volume LII (n. º4) ISSN 0484-811X

Lamego J, Coroadinha A, Simplício A. Bridging the gap between Caco-2 cells and human carboxylesterases. *Toxicology Letters* 2011; 205: S165-S165 doi 10.1016/j.toxlet.2011.05.576

Lamego J, Ferreira P, Cunha B, Coroadinha AS and Simplício AL. Unraveling human carboxylesterase 2 activity-expression mismatch. *Revista Portuguesa de Farmácia* 2011; Volume LII (n. º6) ISSN 0484-811X

Additional Publications

Peer-reviewed Articles

Lamego J, Ferreira P, Nunes S, Matias A, Simplício AL. Comparative determination of carboxylesterase metabolism in whole-cells and in cell lysates. *Toxicology in vitro* 2012; submitted.

Abbreviations

4-MUB 4-Methylumbelliferone

4-MUBA 4-Methylumbelliferyl acetate

4-MUP 4-Methylumbelliferyl phosphate

ABCC1 ATP-binding cassette sub-family C (CTFR/MRP), member 1 (also known as multidrug resistance-associated protein 2 – **MRP1**)

ABCC2 ATP-binding cassette sub-family C (CTFR/MRP), member 2 (also known as multidrug resistance-associated protein 2 – **MRP2**)

ABCG2 ATP-binding cassette sub-family G (WHITE), member 2 (also known as brest cancer resistance protein – **BCRP**)

AcChE Acetylcholinesterase

AcTCh Acetylthiocholine

ADME Absorption, distribution, metabolism and excretion

AMEM Minimum Essential Medium Alpha

ALP Alkaline phosphatase

APC CPT-11 aminopentane carboxylic acid metabolite

ATCC American Type Culture Collection

AU Arbitrary Units

BAP Bovine alkaline phosphatase

BCA Bicinchoninic acid

BES Background electrolyte solution

BNPP Bis-*p*-nitrophenyl phosphate

BuChE Butyrylcholinesterase

BuTCh Butyrylthiocholine

Caco-2 Colon adenocarcinoma 2 cells

CAR Constitutive androstane receptor

CE Capillary electrophoresis

CES Carboxylesterase

CES1 Carboxylesterase 1

CES2 Carboxylesterase 2

CES2-10xHis Recombinant CES2 with an in-frame C-terminal 10× histidine tag

CES3 Carboxylesterase 3

CHO Chinese hamster ovary cells

CO_2 Carbon dioxide

COS-7 African green monkey kidney fibroblast-like cell line

CPT-11 (Irinotecan) (4*S*)-4,11-Diethyl-3,4,12,14-tetrahydro-4-hydroxy-3,14-dioxo-1*H*-pyrano[3',4':6,7]indolizino[1,2-*b*]quinolin-9-yl-[1,4'-bipiperidine]-1'-carboxylic acid ester hydrochloride

CV_r Coefficient of variation of repeatability

CV_i Coefficient of variation of intermediate precision

CYP Cytochrome P450

DMEM Dulbecco's Modified Eagle Medium

DMSO Dimethyl sulfoxide

DMSZ Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures

ECACC European Collection of Cell Cultures

ECVAM European Centre for the Validation of Alternative Methods

EDTA Ethylenediaminetetraacetic acid

Endo H Endoglycosydase H

ER Endoplasmic reticulum

FAH Fumarylacetoacetate Hydrolase

FBS Foetal bovine serum

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

GPx4 Glutathione peroxidase 4

hCES Human carboxylesterase

HEK-293 Human embryonic kidney 293 cells

HEK-293T HEK-293 cells constitutively expressing SV40 large T antigen

HEPES 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)

HIEL CES1 Histidine-isoleucine-glutamic acid-leucine CES1 motif

HPLC High-performance liquid chromatography

Hpt Hours post-transfection

HRP Horseradish peroxidase

HTEL CES2 Histidine-threonine-glutamic acid-leucine CES2 motif

i.d. Internal diameter

IL2RG Common gamma chain or Interleukin-2 receptor subunit gamma

LLC-PK1 Lewis lung carcinoma-porcine kidney 1 cells

IMAC Immobilized metal affinity chromatography

IV Intravenous

IVIVC *In vitro-in vivo* correlation

kDa Kilodalton

KO Knockout

MDCK Mardin-Darby canine kidney cells

MDP Membrane dipeptidase

NaOH Sodium hydroxide

NBT/BCIP Nitro-blue tetrazolium/ 5-bromo-4-chloro-3'-indolyphosphate

NIH National Institutes of Health

NME New molecular entity

NPC CPT-11 primary amine metabolite

ON Overnight

p p-value

p.a. pro analysis

***p*-ABA** *p*-aminobenzoic acid

PALP Human placental alkaline phosphatase

PAMPA Parallel artificial membrane permeability assay

PBPK Physiologically based pharmacokinetic

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PEI Polycation polyethylenimine

P-gp P-glycoprotein (also known as multidrug resistance protein 1 – **MDR1** and ATP-binding cassette sub-family B member 1 – **ABCB1**)

PNGase F Peptide: N-glycosidase F

***p*-NP** *p*-Nitrophenol

***p*-NPA** *p*-Nitrophenyl acetate

***p*O_2** partial pressure of oxygen

PVDF Polyvinylidene difluoride

PVPA Phospholipid vesicle-based permeation assay

PXR Pregnane X receptor; also termed pregnane-activated receptor (SXR) or steroid xenobiotic receptor (SXR)

QEDL CES3 Glutamine-glutamic acid-aspartic acid-leucine CES3 motif

R^2 R-squared coefficient

RAG2 Recombinant Activating Gene 2

RAJI Burkitt's lymphoma-derived cell line

RNase B Ribonuclease B

RPMI Roswell Park Memorial Institute medium

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SN-38 (7-Ethyl-10-hydroxycamptothecin) (4*S*)-4,11-Diethyl-4,9-dihydroxy-1*H*-pyrano[3',4':6,7]indolizino[1,2-*b*]quinolone-3,14(4*H*,12*H*)dione

SNP Single nucleotide polymorphism

TCh Thiocoline

TEER Trans-epithelial resistance

UGT Uridine 5'-diphospho(UDP)-glucuronosyltransferase

UV Ultraviolet

V Volume

V79 Chinese Hamster Fibroblast cell line

Vvm Gas **v**olume flow per unit of liquid **v**olume per **m**inute

W Weight

Units

cm Centimetre

g Times gravity

h Hour

M Molar

mg Milligram

min Minute

mL Millilitre

mM Millimolar

mU Milliunit

ng Nanogram

nm Nanometre

nmol Nanomole

rpm Revolutions per minute

s Second

vvm vessel volumes per minute

µA Microampere

µg Micrograms

µL Microliter

µm Micrometre

µM Micromolar

µmol Micromole

° C Degree Celsius

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SECTION 1

Introduction

Contents

1.1 The fundamentals of carboxylesterases

Carboxylesterases (CESs), generally regarded as hydrolytic enzymes, are involved in the conversion of ester containing compounds into their carboxylic acid and alcohol metabolites. The enzymatic mechanism, through which these enzymes act upon their substrates, has been previously reviewed (Satoh and Hosokawa 2006) and new insights continuously arise through classical enzymatic activity assays as well as more recent techniques such as X-ray crystallography (Redinbo and Potter 2005). Carboxylesterases have been traditionally classified with acetylcholinesterases (AcChE) and butirylcholinesterases (BuChE) as type-B esterases. These enzymes are inhibited by organophosphates, unlike type-C (such as acetylesterases) and type-A hydrolases (such as paraoxonase; Table 1.1). Type-A esterases hydrolyse these compounds, such as paraoxon, but type-C esterases do not interact with them (Aldridge 1993; Liederer and Borchardt 2006).

Table 1.1 Esterases differentiation according with organophosphates hydrolisys/inhibition

Esterase type	Hydrolyse organophosphates?	Are inhibited by organophosphates?
A	Yes	No
B	No	Yes
C	No	No

Aldridge 1993;Liederer and Borchardt 2006.

After oxidative enzymes such as cytochrome (CYP) P450 enzymes and UDP-glucuronosyltransferases (UGTs), esterases are the third major class of enzymes involved in the metabolic clearence of currently administered therapeutic drugs, (Williams et al. 2004; Liederer and Borchardt 2006). Esterases B are additionally classified as belonging to the α/β-hydrolase fold superfamily. α/β-Hydrolases have their secondary structure characterised by an α/β/α sandwich in which five to eight β-sheets form a core connected by α helices (Hotelier et al. 2004). Type-B esterases are serine α/β-hydrolases in which a catalytic triad composed of serine, glutamate and

histidine aminoacids connected by hydrogen bonds is essential for their enzymatic activity (Liederer and Borchardt 2006).

1.1.1 Carboxylesterases across species

Carboxylesterases are ubiquitously present throughout all forms of life, from bacteria to man. Their enzymatic capability has been explored through diverse industrial applications such as organic chemical synthesis and agrochemical industry, being this a very active research field (Jeon et al. 2011). Special attention has been devoted to thermostable carboxylesterases isolated from different *archea*, due to their possible application as industrial biocatalysts (Angkawidjaja et al. 2012). Carboxylesterases role in the metabolism of xenobiotic compounds is well known. By acting as detoxifying agents, they have, for instance, been applied in environmental monitoring (Wheelock et al. 2006). Other functions, however, have been attributed to carboxylesterases. In fungi, best known as feruloyl esterases, they have been implicated in hemicellulose solubilisation (Tartar et al. 2009). In insects, their functions range from insecticide resistance and other detoxifying functions, to pheromone-degrading enzymes, the first being generally intracellular and the second, extracellular (Durand et al. 2012; Claudianos et al. 2006). In plants, they have been described as having important defensive functions without intrinsic catalytic activities (Akashi et al. 2005). In olive (*Olea europaea*) pollen, for instance, they have been identified and implicated in its germination (Rejón et al. 2012).

Being so diverse and widely represented, carboxylesterase family classification is not a trivial task. It has been proposed to divide it into thirteen clades: eight exclusive plant carboxylesterases clades (I to VIII); clade C containing the fungi *Aspergillus nidulans* genes and clades A, B, D and E all containing representatives of microorganisms, other fungi and mammals (Akashi et al. 2005). Although a good effort, this classification has been based in only one hundred and two sequences. Thus, alternative classifications exist. For example, microbial carboxylesterases are

usually classified on the basis of conserved sequence motifs and biological properties, comprising eight families (I to VIII; Jeon et al. 2011). Insect carboxylesterases have been classified into three major classes, subdivided into thirteen clades (Claudianos et al. 2006). The ESTHER database has a comprehensive amount of available α/β-hydrolases gene and protein information from all species. Useful links to other databases as well as other relevant information are also available (Renault et al. 2005).

1.1.2 Looking in more detail to carboxylesterases in mammals

Mammalian carboxylesterases, the most studied, also have their own classification, into five groups (CES1 to CES5), mainly attending to sequence identity (Satoh and Hosokawa 2006). Due to recent advancements in whole genome sequencing, several new *CESs* genes have been unravelled in different species, such as opossum, a marsupial, and primates . Their comparison with the already known *CESs* has been performed (Holmes et al. 2008; Holmes et al. 2009; Williams et al. 2010). This diversity has converged to the proposal of a new nomenclature for mammalian carboxylesterases (Holmes et al. 2010). The newly proposed nomenclature was not followed in this thesis as it is restricted to few mammalian species, posing difficulties in its usage.

Mammalian CESs are intra or extracellularly localised in diverse tissues. Intracellular CESs are usually found inside the endoplasmic reticulum (ER) having a specific retention sequence that interacts with the KDEL receptor (Satoh and Hosokawa 2006) as it happens with human carboxylesterases (hCESs; Satoh and Hosokawa 2006). Nonetheless, carboxylesterase 1 isoform 1 (CES1A1 or CES1_AB119997) has been reported to exist in hepatic cytosol (Tabata et al. 2004). Differences in the number, tissue distribution, substrate selectivity as well as sensitivity towards different inhibitors have been previously reported for different mammals (Williams et al. 2011). For example, mice and rats not only have more CESs

than humans, they also present several secreted forms in the plasma and different substrate specificity: the pranlukast drug was found to be hydrolysed in rats but not in humans (Fukami and Yokoi 2012).

In terms of regulation, mammalian carboxylesterases share similarities with other xenobiotic-metabolising enzymes since they may be induced by similar agents. An example is their induction by chemicals such as phenobarbital, well known to induce CYP enzymes (Satoh and Hosokawa 1998). In the case of down-regulation, hCES1 and carboxylesterase 2 (hCES2) as well as CYP enzymes are suppressed by Interleukin-6 (Yang et al. 2007). Nonetheless, specific inhibitors for CESs have been reported, such as bis-*p*-nitrophenyl phosphate (BNPP) and benzyl (Yoon et al. 2004; Tsurkan et al. 2012). Due to their potential pharmacological application, the search for CESs specific inhibitors is, in fact, an active research field.

Human CES1 has traditionally been the most studied hCES, being the only human form that has, so far, a fully known structure (Bencharit et al. 2003). However, increased attention has been devoted to hCES2 due, for example, to its role in the activation of the anti-cancer prodrug irinotecan (CPT-11) and its potential application in prodrug-activating gene therapies (Yano et al. 2008; Uchino et al. 2008).

1.1.3 Human carboxylesterase 2

Human CES2 is found in different tissues, especially in the liver, intestine, and kidney (Fukami and Yokoi 2012). It constitutes a good example of the aforementioned species variation of CESs. Through a BLAST-P (Altschul et al. 1997) search, the first fifty hits correspond exclusively to eighteen mammalian species (Table 1.2). The search was performed in ESTHER database against all protein sequences available (http://bioweb.ensam.inra.fr/ESTHER/general?what=index), using as query the amino acid sequence of hCES2, which is shown as the first entry of the table (see Appendice Table for the technical details concerning the search). The

sequence identities towards the used query, as well as additional information are provided. These include: protein accession numbers in ESTHER and UniProt databases; alternative protein entries (italicized); protein name and function information available at UniProt database; gene designation (italicized) when existing; GeneBank accession number; level of experimental evidence of protein existence; length of the full protein as well as the last four amino acids. The first non-mammalian hydrolase arising in the search is an uncharacterised protein from *Anolis carolinensis*, an American chameleon (class *Reptilia*), showing 50% amino acid sequence identity to hCES2, whereas hCES1 only appears later, with 46% identity towards the query (See Appendice Table).

Genetic expression of *hCES2* has been well documented, ranging from gene structure to the potential promoters involved in the transcription initiation in different human tissues (Wu et al. 2003). Moreover, 3 trancript variants of the gene (Schiel et al. 2007) as well as several single nucleotide polymorphisms (SNPs) have been documented (Wu et al. 2004; Kubo et al. 2005). Some of these, such as the IVS10-88 (interVening sequence, i.e., intron) SNP, were shown to decrease *hCES2* mRNA expression in colorectal tumours (Marsh et al. 2004).

Table 1.2 CES2 protein across different species

UniProt protein (*gene*)	UNIPROT	ID %	# AA	Last 4 AA	ESTHER	GeneBank	Function
***Homo sapiens* (Human)**							
Carboxylesterase 2 (*CES2*)	O00748 (*A8K367*, *Q4G0E9*)	100	559	HTEL	human-2cxes	Y09616.1	Detoxification of xenobiotics and activation of ester and amide prodrugs. High catalytic efficiency for hydrolysis of cocaine, 4-MUBA, heroin and 6-monoacetylmorphine (Pindel et al. 1997).
***Pan troglodytes* (Chimpanzee)**							
Uncharacterised protein	H2QBA6	97	623	HTEL	pantr-h2qba6	AACZ03102754.1	Hydrolase activity (evp: predicted).
***Gorilla gorilla* (Lowland gorilla)**							
Uncharacterised protein	G3RPG8 (*G3QYW6, G3QYX2*)	95	623	HTEL	gorgo-g3qyw6	-	Hydrolase activity (evp: predicted).
***Pongo abelii* (Sumatran orangutan)**							
Uncharacterised protein (*CES2*)	H2NR54	93	623	HTEL	ponab-h2nr54	-	Hydrolase activity (evp: predicted).
***Nomascus leucogenys* (Northern white-cheeked gibbon)**							
Uncharacterised protein (*CES2*)	G1QVW2	91	606	HTEL	nomle-g1qvw2	ADFV01013581.1	Hydrolase activity (evp: predicted).
***Papio hamadryas* (Hamadryas baboon)**							
Ces2	B5TZ26	90	561	HTEL	papha-b5tz26	FJ147179.1	Hydrolase activity (evp: transcript level; Holmes et al. 2009).
***Macaca mulatta* (Rhesus monkey)**							
Uncharacterised protein	F6UNJ2	88	543	HTEL	macmu-f6unj2	-	Hydrolase activity (evp: predicted).
Putative uncharacterised protein	G7NQ39	88	543	HTEL	macmu-g7nq39	CM001272.1	Hydrolase activity (evp: predicted).
***Callithrix jacchus* (White-tufted-ear marmoset)**							

Uncharacterised protein	F6ZPL6 (*F6Z7R7*)	83	620	HTEL	calja-f6zpl6	ACFV01013743.1, ACFV01013744.1, ACFV01013745.1	Hydrolase activity (evp: predicted).
***Ailuropoda melanoleuca* (Giant panda)**							
Uncharacterised protein (*CES2*)	D2H9C9 (*G1MFN8*)	77	534	HTEL	ailme-d2h9c9	GL192596.1, ACTA01043044.1	Hydrolase activity (evp: predicted; Li et al. 2010).
***Equus caballus* (Horse)**							
Uncharacterised protein (*CES2*)	F7BJ10	77	579	HTEL	horse-f7bj10	-	Hydrolase activity (evp: predicted; Wade et al. 2009).
***Loxodonta africana* (African bush elephant)**							
Uncharacterised protein (*CES2*)	G3TN98	76	554	HTEL	loxaf-g3tn98	-	Hydrolase activity (evp: predicted).
***Canis lupus familiaris* (Dog)**							
Uncharacterised protein (*Ces2*)	F1P6W8	74	585	HTEL	canfa-f1p6w8	-	Hydrolase activity (evp: predicted; Lindblad-Toh et al. 2005).
***Oryctolagus cuniculus* (European rabbit)**							
Uncharacterised protein	G1TZV1	74	558	HTEL	rabit-g1tzv1	AAGW02053044.1	Hydrolase activity (evp: predicted).
Uncharacterised protein	G1SJQ8	74	621	HTEL	rabit-g1sjq8	AAGW02067905.1	Hydrolase activity (evp: predicted).
Uncharacterised protein	G1T6X7	74	558	HTEL	rabit-g1t6x7	AAGW02053044.1	Hydrolase activity (evp: predicted).
Uncharacterised protein	G1TDR0	74	534	HTEL	rabit-g1tdr0	AAGW02067906.1	Hydrolase activity (evp: predicted).
Uncharacterised protein	G1T7P3	73	532	HTEL	rabit-g1t7p3	AAGW02053044.1	Hydrolase activity (evp: predicted).
Uncharacterised protein	G1T7Q5	73	561	HTEL	rabit-g1t7q5	AAGW02053044.1	Hydrolase activity (evp: predicted).
Uncharacterised protein	G1SN51	72	561	HTEL	rabit-g1sn51	AAGW02067906.1 AGW02067907.1	Hydrolase activity (evp: predicted).
Uncharacterised protein	G1TMC5	72	556	HTEL	rabit-g1tmc5	AAGW02053046.1	Hydrolase activity (evp: predicted).

Liver carboxylesterase 2 (*CES2*)	P14943	72	532	HTEL	rabit-2cxes	-	Detoxification of xenobiotics and activation of ester and amide prodrugs (evp: protein level; Ozols 1989).
Uncharacterised protein	G1T6L1	71	528	HTEL	rabit-g1t6l1	AAGW02053044.1	Hydrolase activity (evp: predicted).
***Rattus norvegicus* (Brown rat)**							
Ces2h (*Ces2h*)	Q32Q55	73	558	HTEL	ratno-q32q55	BC107806.1	Carboxylesterase activity (evp: transcript level; Gibbs et al. 2004).
LOC679149 protein	Q4QR68	70	561	HTEL	ratno-q4qr68	BC097486.1	Carboxylesterase activity (evp: transcript level).
Carboxylesterase 5, isoform CRA_a (*Ces2e*)	G3V7J5	70	557	HTEL	ratno-phebest	D50580.1, CH474006.1	Hydrolase activity (evp: predicted; Gibbs et al. 2004).
Ces2c (*Ces2c*)	G3V9D8	70	561	HAEL	ratno-pbcxe	AB010635.1, CH473986.1	Hydrolase activity (evp: predicted; Gibbs et al. 2004).
Carboxylesterase (*Ces2*)	O70177	69	561	HAEL	ratno-sicxe	AB010632	Carboxylesterase activity (evp: predicted).
Ces2g (*Ces2g*)	D3ZXQ0	67	560	HKEL	ratno-d3zxq0	CH473972.1	Carboxylesterase activity (evp: predicted; Gibbs et al. 2004).
Carboxylesterase (Protein Ces2a)	Q8K3R0	66	558	HAEL	ratno-LOC246252	NM_144743, AY034877	Carboxylesterase activity (evp: transcript level; Gibbs et al. 2004).
Ces2i (*Ces2i*)	D3ZE31	66	559	HAEL	ratno-d3ze31	-	Carboxylesterase activity (evp: predicted; Gibbs et al. 2004).
***Cavia porcellus* (Guinea pig)**							
Uncharacterised protein (*CES2*)	H0V5V8	73	568	HTEL	cavpo-h0v5v8	-	Carboxylesterase activity (evp: predicted).
***Bos taurus* (Aurochs)**							
Carboxylesterase 2 (intestine, liver; *CES2*)	Q3T0R6 (*F1MU22*)	72	553	HTEL	bovin-q3t0r6	BC102288.1, AAFC03046191.1	Carboxylesterase activity (evp: transcript level).
***Heterocephalus glaber* (Naked mole rat)**							
Carboxylesterase 2	G5BZE3	72	553	HAEL	hetga-g5bze3	JH172552.1	Hydrolase activity (evp: predicted; Kim et al. 2011).
Carboxylesterase 2	G5BP68	70	570	AAQE	hetga-g5bp68	JH171227.1	Hydrolase activity (evp: predicted; Kim et al. 2011).
Carboxylesterase 2	G5BP66	69	562	HAEL	hetga-g5bp66	JH171227.1	Hydrolase activity (evp: predicted; Kim et al. 2011).
***Mus musculus* (House mouse)**							

Ces2h (*Ces2h*)	F6Z9B9	72	558	HKEL	mouse-Ces2h	AC166833.4, XM_488149.1	Carboxylesterase activity (evp: predicted; Church et al. 2009).
Ces2c (*Ces2c*)	Q91WG0	71	561	HREL	mouse-Ces2c	BC015290.1, AC166833.4	Carboxylesterase activity (evp: transcript level; Furihata et al. 2003).
Uncharacterised protein (*Ces2d-ps*)	D3YWM6	71	558	HREL	mouse-Ces2d-ps	-	Hydrolase activity (evp: predicted; Church et al. 2009).
Ces2b (*Ces2b*)	Q6PDB7	71	556	HTEL	mouse-Ces2b	BC058815.1	Carboxylesterase activity (evp: transcript level; Mural et al. 2002).
Carboxylesterase 5 (Protein Ces2e)	Q8BK48	70	559	HkEL	mouse-Ces2e	XM_134366, BC022148	Carboxylesterase activity (evp: transcript level).
Ces2g (*Ces2g*)	E9PV38	68	560	HKEL	mouse-Ces2g	BC027185.1, BC024548.1, BC026641.1	Carboxylesterase activity (evp: predicted; Church et al. 2009).
Putative uncharacterised protein (*Ces2a*; *Ces6*)	Q3TMR2 (*E9Q3D0*)	67	525	HAEL	mouse-Ces2a	BC024491.1, BC024517.1, BC025537.1	Carboxylesterase activity (evp: transcript level).
***Cricetulus griseus* (Chinese hamster)**							
Carboxylesterase 2	G3IIG3	71	511	HGEL	crigr-g3iig3	JH003006.1	Hydrolase activity (evp: predicted; Xu et al. 2011).
Liver carboxylesterase	G3IIG1	70	561	HKEL	crigr-g3iig1	JH003006.1	Hydrolase activity (evp: predicted; Xu et al. 2011).
Liver carboxylesterase	G3I767	70	535	HKEL	crigr-g3i767.2	JH001411.1	Hydrolase activity (evp: predicted; Xu et al. 2011).
Liver carboxylesterase	G3I766	69	561	HQEL	crigr-g3i766	JH001411.1	Hydrolase activity (evp: predicted; Xu et al. 2011).
Liver carboxylesterase	G3I769	67	545	HAEL	crigr-g3i769:	JH001411.1	Hydrolase activity (evp: predicted; Xu et al. 2011).
***Mesocricetus auratus* (Golden hamster)**							
Carboxylesterase	O35533	70	559	HQEL	mesau-cxest2	D50577	Carboxylesterase activity (evp: trancript level; Sone et al. 1994)
Liver carboxylesterase	Q64419	66	561	HSEL	mesau-cxest	D28566.1	Detoxification of xenobiotics and activation of ester and amide prodrugs. (evp: transcript level; Sone et al. 1994).

ID - identity; #AA – protein length (number of aminoacids); evp – evidence of protein existence (according with UniProt 5 levels – see Apendice).

1.2 The tools to study carboxylesterases

The study of CESs, such as their quantification and differentiation from other enzymes, may be performed through different techniques targeting expression levels, from gene to protein expression, by generating specific primers and antibodies (Sanghani et al. 2003; Morgan et al. 1994). Recently, simultaneous detection and quantification of hCES1 and hCES2 proteins by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was also shown to be possible (Sato et al. 2012). A direct correlation between protein levels and enzyme activity is not always possible for these enzymes. Reports of both inability (Ross et al. 2012) and ability of performing such correlations (Sato et al. 2012) may be found in the literature. Different tools to evaluate CESs activity exist and have been reviewed, such as spectrophotometry, high-performance liquid chromatography (HPLC), and in-gel activity assays (Ross and Crow 2007).

Carboxylesterase-mediated hydrolysis has been thoroughly studied and it is clear that many substrates have recognittion among different CESs as well as with other esterases. For example, *p*-nitrophenyl acetate (*p*-NPA), a classical CESs substrate, is hydrolysed not only by the different CESs but also by cholinesterases (Satoh and Hosokawa 2006). Even pharmacologically relevant compounds known for their higher specificities towards CES2, such as aspirin (Tang et al. 2006), irinotecan (Humerickhouse et al. 2000), and cocaine (Hatfield et al. 2010) are also hydrolysed by BuChE (Li et al. 2005).

Due to this promiscuity in substrate hydrolysis, it is very difficult to differentiate a single carboxylesterase activity in samples containing multiple CESs or other esterases by simple spectrophotometric assays. A classic example is the late demonstration of the absence of CES activity in healthy human plasma through an in-gel activity assay with different substrates and inhibitors for CESs, cholinesterases, and other enzymes (Li et al. 2005). Using a similar approach by combining activity

detection in the presence of certain substrates and inhibitors with protein detection through Western blot, it was recently proposed to be possible to characterise an individual's liver in terms of CES profile (Ross et al. 2012).

A different perspective in CES analysis may arise upon looking to more physiological relevant conditions such as protein localisation and/or activity in whole-living cells, instead of analysing purified enzymes, cell lysates, or tissue homogenates. The importance of addressing how enzymes behave in living cells, where complex networks of protein interactions occur, is detailed in Section 1.3.1. Recent advances in whole-living cell analysis have been made for cytoplasmic membrane anchored enzymes with the use of fluorescent probes (Ferruzza et al. 2012). Evaluating the activity of these types of enzymes is by far an easier task than to quantify the activity of ER localised enzymes, as it is the case of the majority of CESs. Nonetheless, recent advances have been made, such as the development of specific CES fluorescent probes (Wang et al. 2011) that have been applied to live imaging of whole-living cells (Hakamata et al. 2011).

The development of simple, fast, and reliable tools enabling the differentiation and quantification of different CESs expression and activity is thus a very active research field, where some controversial questions still remain. To develop and improve the methodologies, as well as to test new substrates and inhibitors that will also improve already existing methodologies, purified CES enzymes are needed, as they constitute an easier step towards the analysis of more complex samples, such as cell extracts and tissue homogenates. Insights into the latest advances in recombinant CES manufacturing are given in Section 3.

1.3 From drug metabolising enzymes to intestinal permeability models

1.3.1 The importance of seeing both sides - The irinotecan pathway in a human organism as a case study

Notwithstanding the importance of studying purified or recombinant enzymes, it is crucial to understand their behaviour in the most physiologically relevant level, meaning the environment where native enzymes exist. This may mean that studies involving cells and/or organisms may have to be performed. An example involves the metabolism of the chemotherapeutic drug irinotecan. As mentioned above, hCES2 hydrolyses irinotecan into its active metabolite, SN-38. The enzymatic reaction has been characterised with purified enzyme (Humerickhouse et al. 2000), resulting in the determination of important kinetic parameters. Nonetheless, the pathway of intravenous-delivered (IV) irinotecan transport and metabolism in the organism is complex involving the interplay of a complex network of proteins (Figure 1.1) in different cellular compartments. Phase I metabolising enzymes, including CES and CYP3A family members, as well as Phase II enzymes, such as UGTs and the interplay with drug efflux pumps from the ABC family. Both CESs and BuChEs are able to hydrolyse irinotecan, with CESs being more efficient to convert to SN-38. Human CES2 was shown to be one hundred-fold more efficient than hCES1, and hCES3 the least efficient (Humerickhouse et al. 2000). Due to the differences in the hCES1 and hCES2 expression in the liver and intestine, hCES2 accounts for the majority of irinotecan activation in the intestine and kidney while hCES1 has an important function in the activation of this pro-drug in the liver (Hatfield et al. 2011).

Irinotecan is inactivated through oxidation by CYP3A4/5 into aminopentane carboxylic acid (APC), M4, and primary amine metabolite (NPC; Santos et al. 2000; Innocenti et al. 2009). SN-38 is inactivated by UGT Phase II enzymes, namely UGT1A1/6/7/9/10 (de Jong et al. 2007).

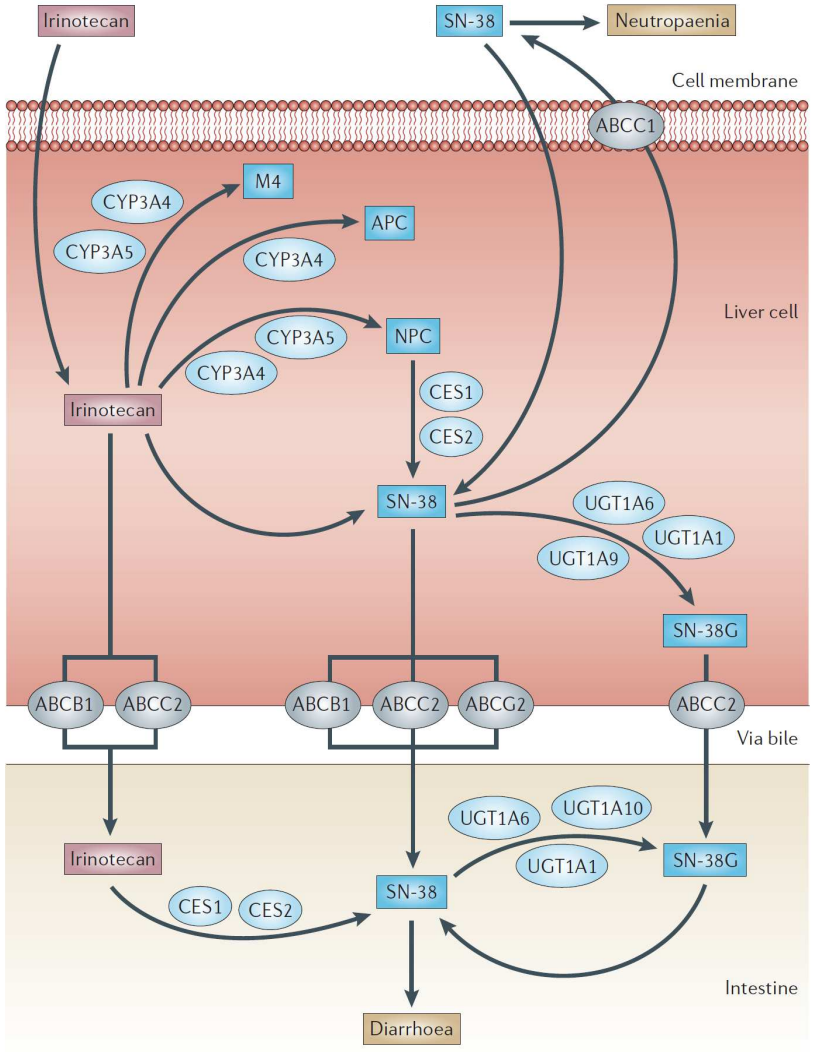


Figure 1.1 Transport and metabolism networks: irinotecan pathway and the role of hCES2. *Irinotecan, IV administered, is metabolised into its active metabolite, SN-38, in liver cells by Phase I enzymes hCES1 and hCES2, and may be secreted throughout the ABCC1 active transporter. Irinotecan may also be oxidised into its inactive metabolites, APC, NPC and M4 by CYP3A enzymes. SN-38 may be further inactivated, through glucoronidation (SN-38G) by Phase II enzymes (UGT1A1, UGTA6, UGTA9, UGTA10) being ultimately eliminated from the organism through bile and urine. Secretion of irinotecan, SN-38 and SN-38G from the cell occurs through active transport (ABCC1, ABCB1, ABCC2 and ABCG2, members of ABC family of drug efflux proteins). Irinotecan is also metabolised in intestinal cells. SN-38 toxicity may include neutropenia and diarrhoea. Adapted and reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Scripture and Figg 2006), copyright (2006) and PharmGKB and Stanford University (Thorn et al. 2003), copyright to PharmGKB (2012).*

Active transport of irinotecan, SN-38, and SN-38G to the extracellular space is facilitated by a few members from the ATP-binding cassette (ABC) family. Irinotecan is transported by ABCC1, ABCB1, and ABCC2; SN-38G is transported by ABCC2, and ABCG2; all of these may be involved in pumping SN-38 (Kweekel et al. 2008).

The chemotherapeutic action of irinotecan is achieved through the binding of SN-38 to topoisomerase I (TOPO-1) during cell division, more specifically, during DNA replication. This complex, formed by SN-38 and TOPO-1, becomes blocked leading to the destruction of the DNA when the replication machinery clashes with it (Marsh and Hoskins 2010). Delayed-type diarrhoea and neutropenia, a decrease in neutrophils increasing the risk of infection (Ammann et al. 2012), are the most common toxic effects of irinotecan and their severity, reaching life-threatening cases, has been linked to some polymorphisms in certain genes, such as *UGT1A1*28* (Marsh and Hoskins 2010; Innocenti et al. 2009; de Jong et al. 2007).

Other examples of the interaction between different metabolic enzymes and efflux transporters have also been studied, such as the case of the interplay between P-glycoprotein (P-gp), multidrug resistance-associated protein 2 (Mrp2), CYP3A, and CES2 in the oral availability of vinorelbine, a pharmaceutical indicated for the treatment of some types of lung and breast cancer. Looking to these interactions in living cells or organisms may be crucial to better understand the reasons behind inter-individual variability in oral dosing (Lagas et al. 2012).

1.3.2 Intestinal permeability and metabolism overview

Oral delivery is still the most common way of dosing drugs to the patients (Buckley et al. 2012). Every drug undergoes a series of transformations inside the organism that depends not only on the type of drug but also on the individual, due to the genetic variability found in humans. Overall, this path is refered to as the ADME (absorption, distribution, metabolism, and excretion) profile of a drug and involves many organs,

such as intestine, liver, and kidney, where different transformations may occur. It may be categorised in different stages, from Phase I-III. Phase I involves the modification of the compounds which may occur through oxidation or hydrolysis, for example. In Phase II, conjugation enzymes usually increase the hydrophilicity of the metabolised compound through the addition of a hydrophilic molecule, such as glucoronic acid, sulphate, or glutathione, to facilitate its elimination. Phase III involves the elimination of the compounds through active transporters (Huynh et al. 2009). Tissue distribution of these proteins has been previously reported (Nishimura and Naito 2006). Being the primary site for xenobiotic absorption and with an important function in drug metabolism (Shen et al. 1997), a special focus is devoted to the small intestine in the following sections.

1.3.2.1 Architecture and function of intestinal epithelium

The intestinal epithelium is found in two, the small and large intestine, of the four segments composing the gastrointestinal tract (GI). In the large intestine, the intestinal epithelium has a flat shape, punctuated with invaginations. In the small intestine, it is composed of projections, the villi, and invaginations, the crypts of Lieberkühn (Figure 1.2). This different morphology is related with different functions of both segments, where the small intestine is mainly responsible for absorption and re-absorption of water is mainly performed by the large intestine. (Rizk and Barker 2012; Dubreuil 2012; Vereecke et al. 2011). The intestinal epithelium protects the internal environment of the organ acting as a selective barrier, due to the tight junctions established between adjacent cells that also contribute to the integrity of the epithelial layer.

There are different types of junctions characterised by their localisation and by the types of proteins involved. From apical to basolateral, one finds tight junctions followed by adheren junctions. These compose the apical junctions, involved in and

regulating the paracellular permeability, a type of transport occurring between cells. Gap junctions and desmosomes are the more basolateral types of junctions (Elamin et al. 2012; Ashida et al. 2011; Gumbiner 1996).

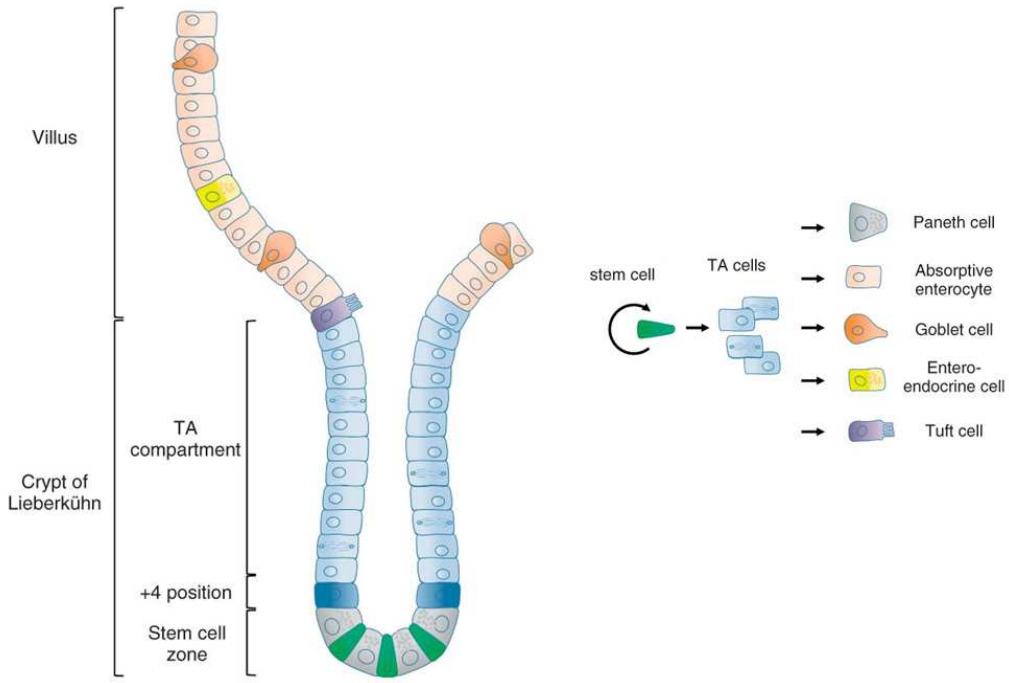


Figure 1.2 Intestinal epithelium architecture and renewal. *Intestinal epithelial cells differentiate as they migrate along the crypt-villus axis. Stem cells, housed at the crypt, give rise to the transient amplifying (TA) cells, which move forward originating the different intestinal epithelial cells. Fully differentiated cells reaching the tip of the villi enter programed cell death (apoptosis) being liberated into the lumen. Paneth cells are the exception cells migrating downwards. Adapted and reprinted with permission from Wiley Periodicals, Inc.: Wiley Interdisciplinary Reviews: Systems Biology and Medicine (Rizk and Barker 2012), copyright (2012).*

Mammalian intestinal epithelia are composed of different differentiated cell types: enterocytes (or absorptive cells), enteroendocrine, goblet, Paneth, Tuft, and M cells. All of these originate from transient amplifying cells (TA) which in turn derive from the stem cells housed at the base of the crypt. These cells differentiate along their migration path across the crypt-villus axis with a rapid renewal of 2-5 days. The exceptions are the Paneth and M cells which migrate towards the base of the crypt

(Dubreuil 2012; Vereecke et al. 2011; Nicoletti 2000). The majority of intestinal epithelium is composed of enterocytes, polarised cells specialised in absorption, bearing microvilli at their apical surface, also called the brush border membrane. Goblet cells are responsible for mucus production and are found both in small and large intestinal epithelia. Paneth cells are specialised in the production of antimicrobial factors being exclusive to the small intestine (Vereecke et al. 2011; Simon-Assmann et al. 2007). Enteroendocrine, Tuft, and M cells are less represented cell populations. The first are responsible for the secretion of diverse hormones thus contributing to the overall homeostasis of the epithelium. The functional significance of Tuft cells remains uncertain (Rizk and Barker 2012). M cells, present in both small and large intestinal epithelia, have defensive roles, being a route for the entry and contact between antigens (such as those from bacteria and virus) and the intestinal immune system (Nicoletti 2000). M cells have also been shown to be able to derive from differentiated absorptive enterocytes. In a curious experimental approach, fully differentiated enterocyte-like Caco-2 cells cultured in the presence of primary lymphocytes were converted into cells having M cell characteristics (Kernéis et al. 1997).

1.3.2.2 Enterocytes – permeability and metabolism

Until reaching the systemic circulation, orally delivered therapeutics must cross the intestinal epithelium followed by the liver, through the hepatic portal vein. The exceptions, those not reaching the liver, are absorbed but directly enter the lymphatic system or are absorbed in the distal rectum (Shen et al. 1997). Oral bioavailability (F_{oral}) corresponds to the fraction of dose that is absorbed (F_a) and not metabolised, crosses the intestinal epithelia to the hepatic portal vein (F_g) and not metabolised in the liver (F_h). Thus, the fraction of the administered drug that effectively reaches its site of action corresponds to the product of these three variables, a process also

known as first-pass biotransformation, where several metabolic enzymes and transporters are invovled (Thelen and Dressman 2009).

The players involved in transport and metabolism in enterocytes have been extensively studied through several techniques. Different layers of expression and function, from genomics to metabolomics and transportomics have been thoroughly evaluated under various physiological conditions including inflammation (Stegmann et al. 2006; Fleet 2007; Béaslas et al. 2008; Romero-Calvo et al. 2011). Orally delivered xenobiotics, such as nutrients and drugs, have various routes to cross the intestinal epithelium (Figure 1.3).

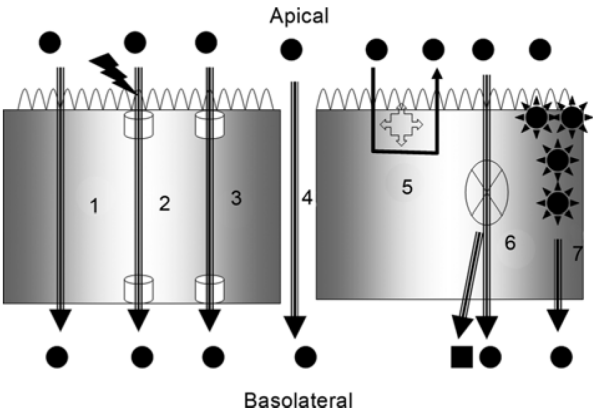


Figure 1.3 Intestinal absorption routes. *Xenobiotic compounds may cross the intestinal epithelium through (1, 2, 3, 5, 6 and 7) or in between enterocytes (4). 1. Transcellular passive transport. 2. Active transport. 3. Facilitated passive diffusion. 4. Paracellular passive transport. 5. Efflux active transport. 6. Metabolic reaction. 7. Endocytosis. Reprinted with permission from Springer (Springer, Part of Springer Science+Business Media): The AAPS Journal (Balimane et al. 2006), copyright (2012).*

Intestinal absorption may occur through the intestinal cells or between cells and may involve the interplay of both transport proteins as well as metabolic enzymes. Passive absorption most commonly occurs through the cells, known as transcellular transport, where compounds are able to cross the cell membrane due to an adequate lipophylicity. Transport across channel proteins without energy expenses, known as facilitated diffusion or passive carrier-mediated absorption

(uniport), is another possibility. Absorption can also occur between cells, known as paracellular transport, where compounds transverse the junctions between cells.

Drugs and other compounds may also be transported through the cells by endocytosis and active primary transport, utilising ATP, or active secondary transport, such as coupled carrier-mediated transport (symport or antiport). Efflux transporters are examples of active primary transporters and counter the apical to basolateral transport by the active transport of compounds towards the lumen of the intestine. They are members of the ABC family such as the breast cancer resistance protein (BCRP), P-gp and multidrug resistance-associated protein (MRP). Some Phase I metabolic enzymes, such as those from CYP and CES families, may metabolise the transported compound thus interfering with its absorption rate (Balimane et al. 2006; Buckley et al. 2012; Alberts et al. 2002). An example is the interplay between CYP3A metabolic enzymes and P-gp transport, where several drugs are substrates of both proteins (Christians et al. 2005; van Waterschoot and Schinkel 2011).

The liver is the organ traditionally regarded as the primary site of drug metabolism. Nonetheless, the importance of intestinal metabolism should not be overlooked. It has been demonstrated that the majority of the metabolic enzymes found in liver cells are also present in the enterocytes. The enzymes found in small intestine have been extensively reviewed and include Phase I (CYP enzymes, with CYP3A4 being the most abundant; esterases; epoxide hydrolase; alcohol dehydrogenase) and Phase II (UGTs, sulfonotransferases, acetyl transferases, and glutathione S-transferases) metabolic enzymes (Bonnefille et al. 2011; Thelen and Dressman 2009; Lin et al. 1999; Shen et al. 1997).

1.4 Tools to study intestinal permeability and metabolism

Several tools are available to evaluate intestinal permeability of drugs. Ranging from *in vivo*, *in situ*, *ex vivo*, *in silico*, and *in vitro* assays, they have been extensively reviewed in the literature (Cheng et al. 2008; Buckley et al. 2012; Volpe 2010; Geerts et al. 2011). The suitability of any given experimental model may be evaluated according to the accuracy of the *in vitro-in vivo* correlation (IVIVC) (Volpe 2010). No perfect model exists and the choice of which model(s) to use must rely on the knowledge of their main advantages and limitations. Several authors have proposed different strategies and outcomes on how to perform this choice (Fagerholm 2007; Christensen et al. 2012). Under the scope of this thesis, a brief overview of the possible approaches is performed with a special emphasis on *in vitro* models and Caco-2 cells.

1.4.1 *In vivo*

The usage of experimental or laboratory animal models constitutes the only whole living organism approach possible, besides the clinical studies performed with humans. Rat, dog, monkey, sheep, mouse, and pig are some of the most commonly used animals (Harrison et al. 2004; Fagerholm 2007; Cheng et al. 2008).

In addition to the regulatory, ethical and economic constraints inherent to the use of animals (please refer to Section 1.4.6) another severe limitation arises from the demonstrated species differences that may affect the accuracy of the IVIVC (Crow et al. 2007; Williams et al. 2011). In fact, species differences may impact more than the extrapolation of absorption data to humans. An unfortunately notorious example is thalidomide, a mild sleeping pill that reduced morning sickness and was commercialized in the mid 1960's. It caused the birth of several thousand impaired children although no defects had been previously noticed in rat newborns. It was later

discovered to impair the correct development of rabbit foetuses (Harrison et al. 2004).

1.4.2 *In situ*

In the perfusion *in situ* technique, usually, a segment of the intestine of a numbed animal is perfused with a drug solution containing a predetermined concentration. By measuring the amount of drug that leaves the segment in the original or metabolised form, one may indirectly determine absorption. Different techniques were developed such as open, semi open, or closed perfusion (Lennernäs 1998; Volpe 2010).

Perfusion models constitute the best approximation to the anatomy found in the living organ but require the usage of animals, their manipulation through anaesthesia and surgery, thus being an invasive, time consuming, and low throughput technique (Volpe 2010; Harrison et al. 2004). Resorting to animals, it shares the limitations of *in vivo* assays, mentioned above, using animal experimental models.

1.4.3 *Ex vivo*

In explant cultures, portions of the GI tissues are removed and cultured according to different methods and culture techniques (Randall et al. 2011). Two examples are the everted gut sac model and the diffusion chamber. In the first, a portion of everted intestine is filled, tied up and placed in a chamber containing the drug solution. The permeation of the drug is determined by measuring how much appears inside the sac. Different animals have been used for the application of this technique, with rat being the most common (Volpe 2010; Alam et al. 2012). In the diffusion chamber method, a portion of excised tissue is opened and cultured as a single layer in the interface of two chambers. The absorption rate is determined by measuring the amount of drug, placed in the apical chamber, found in the basolateral chamber (Volpe 2010).

Human or non-human animal tissue diffusion models maintain some of the characteristics encountered in the gut, such as the maintenance of the mucus layer. Nonetheless, several limitations may occur in this system such as the limited viability of the tissue and the stirring conditions. Underestimation of drug transport may occur due to the insufficient removal of the *muscularis mucosa*, the inner most layer of the mucosa (composed of the epithelium, *lamina propria*, and *muscularis mucosa*) and the region of the intestine that faces the lumen (Volpe 2010; Randall et al. 2011; Alam et al. 2012).

1.4.4 *In silico*

Computer software to perform physiologically-based pharmacokinetic (PBPK) modelling has been suggested as one of the best possibilities towards animal reduction for predicting drug ADME profiles (Harrison et al. 2004). Different predictive models exist and their potential have been previously reviewed (van de Waterbeemd and Gifford 2003; Theil et al. 2003). Commercially available software including SimCYP® Population-based ADME simulator and GastroPlus™ are examples of the existing possibilities to perform PBPK modelling that are already being used by pharmaceutical companies, such as Roche (Heikkinen et al. 2012).

There is an increased acceptance of these types of studies due to their associated advantages: the costs are inexpensive compared to *in vivo* studies; they have been reported to provide qualitatively accurate predictions, despite the need for improved quantitative accuracy (Jacob et al. 2009); and they allow time savings (Heikkinen et al. 2012). Relying on available parameters, obtained through both *in vivo* and *in vitro* experimentation, a general disadvantage is the quality of the existing data used to perform the modelling studies (Harrison et al. 2004; Fagerholm 2007). Another disadvantage of this approach has been the lack of validated prediction of

intestinal metabolism but different researchers are working towards improving it (Heikkinen et al. 2012).

1.4.5 *In vitro*

1.4.5.1 Non-cellular models

Non-cellular models constitute simple, automatable, and highthroughput adaptable systems. They are usually less expensive and faster than cellular *in vitro* models, due to the avoidance of the culturing times, allowing larger pH ranges and drug concentrations. They are, however, unsuited for active transport studies and lipophilic compounds usually face membrane retention problems. Examples of these systems are the parallel artificial membrane permeability assay (PAMPA) and the phospholipid vesicle-based permeation assay (PVPA). The first is obtained through the coating of a hydrophobic filter, in 96-well plates, with an organic solution containing lecithin/phospholipids. PVPA is composed of a layer of liposomes in a porous filter where the pores are also filed with liposomes. These have been reported as the most effective systems for passive absorption studies through the transcellular route. Higher accuracy has been attributed to the PVPA method due to the avoidance of organic solvents and an apparent negligible unstirred water layer (Balimane et al. 2006; Volpe 2010; Buckley et al. 2012).

1.4.5.2 Cellular models

Cellular models exist for the study of intestinal absorption and have been recently reviewed (Buckley et al. 2012; Sarmento et al. 2012). Primary cultures of enterocytes may be obtained through the differentiation of intestinal embryonic epithelial cells or isolated from adult human intestine. The first human villus-like primary culture of differentiated enterocytes (PCDE), able to survive for ten to twelve days in culture, was reported in 1998 (Perreault and Beaulieu 1998). Several limitations have impaired the routine use of normal human enterocyte primary cell lines, such as their limited

survival time, highly differentiated state, bacterial contamination, overgrowth of other cells from the tissue including mesenchymal cells, and very complex protocols for cell culturing (Simon-Assmann et al. 2007; Chougule et al. 2012). Advances in this field have been recently reported, in terms of improvements on reproducibility and less complex isolation methods (Chougule et al. 2012). The application of these new advances in absorption studies is yet to be performed.

The most common cell culture models are immortalized intestinal epithelial cell lines from human or non-human animal origins (Simon-Assmann et al. 2007; Beaulieu and Ménard 2012; Chougule et al. 2012). Other cell culture models are non-transformed intestinal epithelial cells, such as the rat small intestine epithelia (IEC) cell line, able to differentiate when co-cultured with foetal intestinal mesenchyme (Simon-Assmann et al. 2007). Animal or human derived cellular models enable the study of different types of permeability, such as passive diffusion (both transcellular and paracellular) and active transport including efflux mechanisms. A main advantage is the possibility to automate screens, thus constituting highthroughput techniques. Nonetheless, some of their pitfalls are the variability found between laboratories (inter-laboratory), the lack of expression of several transport proteins as well as a mucus layer, the nonspecific binding of compounds to the cells and to the devices used in cell culturing, and the poor predictability for those compounds crossing the cell by carrier-mediated transport or via the paracellular route (Balimane et al. 2006; Volpe 2010).

Table 1.3 summarises the most common cell lines used for intestinal absorption studies, all of them able to form polarised epithelium–like cells.

Table 1.3 Advantages (+) and limitations (-) of immortalized intestinal epithelial cell lines

Cell line	Species / Tissue	Characteristics
Caco-2	Human / Colon adenocarcinoma	**+** Human origin; spontaneous differentiation becoming enterocyte-like cells; domes formation in culture; expression of eflux transporters (e.g. P-gp, MRP2, BCRP).
		- Tumour origin thus bearing mutations in several genes; inter-laboratory variability; heterogeneous cell line; time-consuming (differentiation through a 21-days period); requires regular medium change; possibility of false negative results in drug permeability, due to lower expression of some carrier-mediated transporters and tighter junctions (paracellular transport), comparing with enterocytes; lack CYP3A4 expression to significant levels (high in enterocytes); low expression of hCES2 and high expression of hCES1 (opposite of what is found in enterocytes).
HT-29	Human / Colon adenocarcinoma	**+** Human origin; differentiation becoming enterocyte-like cells under certain conditions (e.g. glucose-deprivation, glucose substitution by galactose, inosin or uridine as carbon source). Under certain culture conditions they differentiate into goblet cells, enabling drug absorption studies in the presence of a mucus layer.
		- Tumour origin thus bearing mutations in several genes; lower expression of brush-border enzymes than enterocytes or Caco-2 cells.
MDCK	Canine / Kidney	**+** Shorter culturing times (3 days); Good correlation with Caco-2 cells in passive transcellularly transported compounds; Ameanable for cell transfections.
		- Non-human origin; minimal metabolic activity (may be advantageous if metabolism is not a focus of the study); low expression of ABC transporters (may be advantageous; e.g., avoid P-gp efflux).
2/4/A1	Rat / Foetal intestine	**+** More suitable for paracellular studies due to leakier monolayers formation.
		- Non-human origin; temperature sensitive.
LLC-PK1	Pig / Kidney	**+** Ameanable for cell transfections.
		- Non-human origin; Low expression of certain drug transporters (may be advantageous).

Balimane and Chong 2005; Balimane et al. 2006; Cheng et al. 2008; Irvine et al. 1999; Buckley et al. 2012; Sarmento et al. 2012; Simon-Assmann et al. 2007; Ungell 2004; Volpe et al. 2010.

Caco-2 cells, isolated from a colon adenocarcinoma (Fogh et al. 1977), were shown to spontaneously differentiate in culture (Pinto et al. 1983). Since their first characterisation studies (Ramond et al. 1985; Rousset 1986; Hidalgo et al. 1989), they have become the most well-established cell model for intestinal permeability in addition to their application in intestinal differentiation studies. Sub-clones of this cell line were selected with TC-7 being a more homogeneous Caco-2 cell line, leading to higher consistency in the obtained results, thus reducing their variability (Simon-Assmann et al. 2007; Buckley et al. 2012). In Section 1.5, additional attention is devoted to Caco-2 cells.

HT-29, isolated in 1964 by Fogh, was the first human colon carcinoma cell line established. Curiously, this cell line has the ability to differentiate into different cell types depending on the culture conditions: under glucose deprivation, it acquires enterocyte-like characteristics; in serum-free culture, half of the population acquires goblet cells characteristics; upon long-term culture in the presence of sodium butyrate the cells differentiate into goblet cells and the same happens in the presence of intermediate (10^{-6}-10^{-5} mol.L^{-1}) concentrations of the drug methotrexate (MTX); in the presence of high concentrations (10^{-4}-10^{-3} mol.L^{-1}) of MTX, only enterocyte-like cells are found; if low concentrations (10^{-7} mol.L^{-1}) of this drug are present in the culture media, the population becomes half enterocyte-like and half globet-like. This plasticity has led to the isolation of different HT-29 sub-clones, such as the HT29-MTX mucous secreting cell line (Simon-Assmann et al. 2007).

Mardin-Darby canine kidney (MDCK) cells and Lewis lung carcinoma-porcine kidney 1 (LLC-PK1) are cell lines commonly used to assess permeability of xenobiotics (Balimane and Chong 2005). MDCK cells were isolated in 1958 by Mardin and Darby and were immortalized through long-term *in vitro* culture (Bruyneel et al. 1990). LLC-PK1 were isolated in 1976 and shown to be able to form dome-like structures (Hull et al. 1976). Special attention should be devoted to these cell lines as well as to the

2/4/A1, a rat foetal intestinal cell line conditionally immortalized with a temperature-sensitive SV40 large T antigen (Tavelin et al. 1999), due to possible species differences (Balimane and Chong 2005). 2/4/A1 cell line has been proposed as representative for studying paracellular transport; however, differences in paracellular pore size distribution have been reported in comparison with what is found in enterocytes (Ungell 2004; Linnankoski et al. 2010).

One of the critics of working with isolated single-cell cultures is the lack of physiological relevance due to the lack of interplay between cells that is found *in vivo*. As detailed in Section 1.3.2, the small intestine epithelium is composed of different cell types. Strategies to account for this diversity *in vitro* have been developed such as the co-culture of Caco-2 and HT-29, Caco-2 and Burkitt's lymphoma-derived cell line (Raji), and even a triple co-culture system with these three cell lines (Sarmento et al. 2012). Raji cells are a human B-cell reference line, isolated in 1964 from a patient suffering from lymphoma. Curiously, it is on the basis of the discovery and isolation of the Epstein-Barr virus (Pulvertaft 1964; Wu et al. 2004b; Karpova et al. 2005). As mentioned in Section 1.3.2, lymphocytes have the ability to convert Caco-2 into M cells. The co-culture of Caco-2 with Raji cells allows a better mimic of follicle-associated epithelium, regions of the intestinal epithelium containing enterocytes, M, and goblet cells (des Rieux et al. 2007; Sarmento et al. 2012). Some other co-culture strategies trying to mimic the relationships between cells, even from different tissues in the organism, have already been reported such as the co-culture of enterocyte-like Caco-2 cells, with hepatocyte-like HepaRG cells (Rossi et al. 2012).

1.4.6 The need for improved *in vitro* models

As demonstrated in the previous sections, where the main limitations of the currently existing methods were presented, there are several scientific reasons pointing to the need to improve them to reduce the use of animal models. In fact, it was previously

stated that the extent of *in vivo* experimental animals' usage is a consequence of both regulatory constraints and the limitations in currently available *in vitro* models (Schroeder et al. 2011).

Achieving better *in vitro* tools to test drugs and other xenobiotics alleviates ethical and economic concerns. The ethical reasoning behind the need of adjusting the usage of animal models have led to the adoption by the European regulatory authorities of the 3R policy: Replace, Reduce, or Refine laboratory animal use when possible (Wells 2011; Schiffelers et al. 2012). This is included in the measures of the new European Directive 2010/63/EU that will be in effect on the 1st of January of 2013 (Wells 2011). Aligned with the political regulations, the Scientific Advisory Committee of European Centre for the Validation of Alternative Methods (ECVAM) has contributed to the development, promotion, and adoption of new or improved alternatives to *in vivo* methods (Huynh et al. 2009). Although there is an increased pressure to minimise the usage of animal models, its prohibition in the drug development process is not envisioned in the near future (Schroeder et al. 2011). However, the Cosmetics industry is regulated by different legislation, and the usage of animals for testing final products has been prohibited in Europe since 2004. The banning is expected to be extended to cosmetic ingredients, at least for certain assays, in the near future, providing that new or improved *in vitro* testing are developed and validated (Schroeder et al. 2011).

In economic terms, there is also the need to rethink and readjust the use of experimental animals in the process of drug discovery and development, for example. Latest estimations for the costs involved in the research and development (R&D) process of developing one marketable new molecular entity (NME) required investing US$ 1.78 billion to complete the process (Paul et al. 2010). In 2003, the estimations were US$ 403 million (DiMasi et al. 2003), and although differences between the estimation methods may be arguable, there is no doubt concerning the escalation of

the needed investment. These have been mainly attributed to the increase in the costs of clinical trials, as well as in costs involved in animal experimentation. Although clinical trials constitute the highest burden in the entire process, the costs attributed to animal testing are also a reason for concern (Dickson and Gagnon 2004). Therefore, improved *in vitro* models may decrease the investment in the development of new drugs.

1.5 Caco-2 cell line

Caco-2 cell line is one of the most used *in vitro* model for drug absorption prediction and is widely used by the pharmaceutical industry (Buckley et al. 2012) since it is accepted and recommended by the North-American regulatory authorities, the Food and Drug Administration (FDA), and the European authorities, the European Medicines Agency (EMA). Nonetheless, and as mentioned in the previous sections, this model has some limitations related with its intrinsic characteristics as well as the practiced cell culture methods.

Caco-2 cells are known to be hypertetraploid (Pinto et al. 1983), having on average 89 (84-98) chromosomes. Spectral karyotyping revealed fourteen aberrant chromosomes in the Caco-2 karyotype (Melcher et al. 2002).

Caco-2 cells are known to express both colonocyte and enterocyte phenotypes (Engle et al. 1998). Special attention has been devoted to the changes in gene expression occurring throughout the differentiation period. Using transcriptome analysis and comparing both enterocytes and colonocytes, it was shown that until reaching confluence, Caco-2 share mRNA profile similarities with both cell types, and thereafter becoming more and more similar to enterocytes (Anderle et al. 2003). Moreover, it was also found that upon differentiation, down-regulation of cell cycle, transcription and protein metabolism associated genes occured whereas the expression of cell adhesion, iron transport, and Phase II metabolism genes increased

(Bédrine-Ferran et al. 2004; Chopra et al. 2010). Changes in post-translational modification profiles, namely due to differences in glycosyltransferase expression, were also reported upon differentiation of the cells (Brockhausen et al. 1991) as well as in the metabolic profile of the cells, as evaluated through nuclear magnetic resonance (NMR; Lee et al. 2009).

One of the main disadvantages with Caco-2 cells is the intra- and inter-laboratory variability found in the results. The factors on the basis of this variability were previously studied (Zucco et al. 2005; Hayeshi et al. 2008; Prieto et al. 2010; Roth 2012). Table 1.4 shows some examples of each of these factors. So far, there is no standard operating procedure to work with these cells although several methods and conditions aiming to achieve the best culturing conditions were proposed (Hubatsch et al. 2007; Prieto et al. 2012; Natoli 2012). One may envision the best approach to be exploring the provided culturing methods throughout the literature, establish a protocol, and consistently reproduce it using a well characterised Caco-2 cell line in a specific and characterised passage interval. The characterisation of the cells may be achieved through protein and mRNA analysis or the use of reference compounds during permeability experiments. Based upon this notion, it was demonstrated that independent of cell source and culturing conditions, Caco-2 is the *in vitro* cell model genetically closest to human enterocytes (Christensen et al. 2012).

Recently, advances towards the achievement of more relevant physiological conditions were performed by showing that Caco-2 cells in 2-D cultures are able to differentiate in inserts containing only serum in the basolateral chamber (Ferruzza et al. 2012) and these cells are able to grow and differentiate in 3-D cultures (Elamin et al. 2012).

Table 1.4 Examples of different factors affecting Caco-2 variability

Variability factor	Examples
Cell source	Different cell banks provide Caco-2 cells from different depositers causing inter-laboratory variability in the results even when using intra-laboratory standard culturing conditions (Hayeshi et al. 2008). - DMSZ bank; cat.# ACC 169: deposited by Prof. A. Bacher, Technical University of Munich, Munich, Germany (http://www.dsmz.de/catalogues/catalogue-human-and-animal-cell-lines.html). - ECACC bank; cat.# 09042001: from ATCC, 1985; cat.# 86010202: deposited by Dr. J Clarke, AVRI, Pirbrigh (http://www.hpacultures.org.uk/collections/ecacc.jsp). - ATCC bank; cat.# HTB-37™: deposited by J Fogh, 1977; cat.# CRL-2102™: deposited by MD Peterson and M Mooseker (http://www.lgcstandards-atcc.org/).
Passage number	- Influences the expression of efflux proteins: MDR1 expression was lower in Caco-2 cell above passage 45 in comparison with passage 30 (Siissalo et al. 2007). - Influences different functional properties: TEER; cell proliferation rate, and multilayered areas increase at higher passages. Sucrose-isomaltose expression increases and alkaline phosphatase activity decreases also at higher passages (Sambuy et al. 2005).
Seeding inocula during passaging	- Variable growth rates reported; should be adjusted to sub-culturing once a week (Zucco et al. 2005). 80% considered as a good confluence for sub-culturing but recent reports argue lowering to 50% to avoid heterogeneus growth conditions among the cells (Natoli et al. 2011).
Seeding innocula for differentiation	- Wide range of reported seeding densities (10^3 to 10^5 cell.cm^{-2}). Intermediate seeding densities (6 x 10^4 cell.cm^{-2}) reported as suitable for 21-day differentiation. Higher or lower densities reported as negatively influencing P-gp expression. Higher seeding densities may lead to multilayer formation (Sambuy et al. 2005).
Medium	- Different medium reported from AMEM, DMEM to RPMI and different FBS percentages. Better performance was reported with DMEM using 10% FBS (Zucco et al. 2005). Usually additional supplementation with 1% NEAA is performed. - Caco-2 originally from same source shown to have significant differences in drug metabolising enzymes when cultured in AMEM or DMEM (Roth et al. 2012). - Hygromycin B antibiotic impacts cell adherence, monolayer integrity and cell morphology (Crespi et al. 1996; Rodolosse et al. 1998).
Incubation conditions	- 37 °C is the standard temperature used; reported CO_2 % varies from 5-10%; it should be adjusted to the concentration of sodium bicarbonate in the medium to achieve pH 7.4 according to Henderson-Hasselbach equation: pH = 6.1 + log (52 * mg/ml $NaHCO_3$ / %CO_2 -1) (Castilho et al. 2008).
Filter support	- Filter material and pore size influence Caco-2: polycarbonate and pore diameter of 0.4 µm reported as better for monolayer achievement (Hubatsch et al. 2007). Pore size has been reported as impacting dome formation (Ramond et al. 1985).

Cat.# - catalogue number; DMSZ - Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures; ECACC - European Collection of Cell Cultures; ATCC - American Type Culture Collection; AMEM - Minimum Essential Medium Alpha; DMEM - Dulbecco's Modified Eagle's Medium; RPMI - Roswell Park Memorial Institute medium; FBS – Foetal bovine serum; TEER - Trans-epithelial resistance. NEAA – Non-essential aminoacids.

To improve the intrinsic limitations of Caco-2 cells (presented in Section 1.4.5) several strategies have been employed to increase or decrease the expression of various proteins. Using chemical agents such as 1-alpha,25-dihydroxyvitamin-D3, the CYP3A4 expression levels were increased (Schmiedlin-Ren et al. 2001). Using BNPP, an inhibitor of CESs, a model for permeability evaluation without active CESs was achieved (Ohura et al. 2010). Genetic manipulation of Caco-2 cells has also been attempted, not only for the up-regulation of genes, but also for their knockdown, such as the case of Caco-2 transduction with small hairpin RNA (shRNA) targeting P-gp (Li et al. 2011).

1.6 Thesis goals

The main goals of the work developed and presented in this thesis were to enlarge the fundamental knowledge on hCES2 and to improve the Caco-2 cell model by increasing the levels of this enzyme, which is crucial in the metabolism of ester containing drugs and prodrugs.

To achieve the proposed goals, a step-wise approach was followed, where a journey from analytical chemistry to protein manufacturing and cell line development culminates in the opening of several new hypothesis and challenges for this research field, discussed in greater detail in the last section.

The first challenge in this journey, described in Section 2, was the difficulty of studying the activity of hCES2 in biological samples. The existing techniques allowing the differentiation and quantification of the activity of hCES2 were highly time- and sample-consuming. Our first goal was to develop a tool enabling to differentiate and quantify hCES2 activity in different biological samples rapidly and with low sample consumption, for a proper study of this enzyme.

The second challenge faced was the unavailability of a purified hCES2 from human cells. Although an hCES2 form was available from mouse myeloma cells, our goal was to further investigate the properties of hCES2 produced and purified from a human origin. To achieve this goal, a pioneering strategy, described in Section 3, was followed using human embryonic kidney (HEK-293T) cells.

The combined knowledge acquired through the work developed allowed tackling the third challenge: the improvement of the most widely used intestinal permeability model, the Caco-2 cell line. Although used by both academia and industry and accepted by pharmaceutical regulatory authorities, this cell model has weaknesses. Our third goal, detailed on Section 4, was to overcome one of these constraints, the low expression of hCES2 in comparison with human enterocytes.

Overall, the work reported herein impacts different research fields ranging from fundamental to applied biology and biotechnology as well as analytical chemistry, specifically contributing to the fields of xenobiotic metabolising enzymes and relevant preclinical *in vitro* models.

1.7 References

Alam MA, Al-Jenoobi FI, Al-Mohizea AM (2012) Everted gut sac model as a tool in pharmaceutical research: limitations and applications. *J Pharm Pharmacol* 64: 326-336.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the Cell. 4th edition. *New York: Garland Science*; 2002. Carrier Proteins and Active Membrane Transport. Available from: http://www.ncbi.nlm.nih.gov/books/NBK26896/

Aldridge WN (1993) The esterases: perspectives and problems. *Chem Biol Interact* 87: 5-13.

Akashi T, Aoki T, Ayabe S (2005) Molecular and biochemical characterization of 2-hydroxisoflavanone dehydratase. Involvement of carboxylesterase-like proteins in leguminous isoflavone biosynthesis. *Plan Physiol* 137: 882-891.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389-3402.

Ammann RA, Tissing WJ, Phillips B (2012) Rationalizing the approach to children with fever in neutropenia. *Curr Opin Infect Dis* 25: 258-265.

Anderle P, Rakhmanova V, Woodford K, Zerangue N, Sadée W (2003) Messenger RNA expression of transporter and ion channel genes in undifferentiated and differentiated Caco-2 cells compared to human intestines. *Pharm Res* 20: 3-15.

Angkawidjaja C, Koga Y, Takano K, Kanaya S (2012) Structure and stability of a thermostable carboxylesterase from the thermoacidophilic archaeon *Sulfolobus tokodaii*. *FEBS J* 279: 3071-3084.

Ashida H, .Ogawa M, Kim M, Mimuro H, Sasakawa C (2011) Bacteria and host interactions in the gut epithelial barrier. *Nat Chem Biol* 8: 36-45.

Balimane PV, Chong S (2005) Cell culture-based models for intestinal permeability. A critique. *Drug Discov Today* 10: 335-343.

Balimane PV, Han YH, Chong S (2006) Current industrial practices of assessing permeability and P-glycoprotein interaction. *AAPS J* 8: E1-E13.

Béaslas O, Torreilles F, Casellas P, Simon D, Fabre G, Lacasa M, Delers F, Chambaz J, Rousser M, Carrière V (2008) Transcriptome response of enterocytes to dietary lipids: impact on cell architecture, signalling, and metabolism genes. *Am J Physiol Gastrointest Liver Physiol* 295: G942-G952.

Beaulieu JF, Ménard D (2012) Isolation, characterization, and culture of normal human intestinal crypt and villus cells. *Methods Mol Biol* 806: 157-173.

Bédrine-Ferran H, Le Meur N, Gicquel I, Le Cunff M, Soriano N, Guisle I, Mottier S, Monnier A, Teusan R, Fergelot P, Le Gall JY, Léger J, Mosser J (2004) Transcriptome variations in human Caco-2 cells: a model for enterocyte differentiation and its link to iron absorption. *Genomics* 83: 772-789.

Bencharit S, Morton CL, Xue Y, Potter PM, Redinbo MR (2003) Structural basis of heroin and cocaine metabolism by a promiscuous human drug-processing enzyme. *Nat Struct Biol* 10: 349–356.

Bonnefille P, Sezgin-Bayindir Z, Belkhelfa H, Arellano C, Gandia P, Woodley J, Houin G (2011) The use of isolated enterocytes to study Phase I intestinal drug metabolism: validation with rat and pig intestine. *Fundam Clin Pharmacol* 25: 104-114.

Brockhausen I, Romero PA, Herscovics A (1991) Glycosyltransferase changes upon differentiation of Caco-2 human colonic adenocarcinoma cells. *Cancer Res* 51: 3136-3142.

Bruyneel EA, Debray H, De Mets M, Mareel MM, Montreuil J (1990) Altered glycosylation in Madin-Darby canine kidney (MDCK) cells after transformation by murine sarcoma virus. *Clin Exp Metastasis* 8: 241-253.

Buckley ST, Fischer SM, Fricker G, Brandl M (2012) *In vitro* models to evaluate the permeability of poorly soluble drug entities: challenges and perspectives. *Eur J Pharm Sci* 45: 235-250.

Castilho LR, Moraes AM; Augusto EFP, Butler M. Animal Cell-Technology: From Biopharmaceuticals to Gene Therapy. 1st edition. *Taylor & Francis Group*; 2008.

Cheng KC, Li C, Uss AS (2008) Prediction of oral drug absorption in humans – from cultured cell lines and experimental animals. *Expert Opin Drug Metab Toxicol* 4: 581-590.

Christensen J, El-Gebali S, Natoli M, Sengstag T, Delorenzi M, Bentz S, Bouzourene H, Rumbo M, Felsani A, Siissalo S, Hirvonen J, Vila MR, Saletti P, Aguet M, Anderle P (2012) Defining new criteria for selection of cell-based intestinal models using publicly available databases. *BMC Genomics* 13: 274.

Christians U, Schmitz V, Haschke M (2005) Functional interactions between P-glycoprotein and CYP3A in drug metabolism. *Expert Opin Drug Metab Toxicol* 4: 641-654.

Chopra DP, Dombkowski AA, Stemmer PM, Parker GC (2010) Intestinal epithelial cells *in vitro*. *Stem Cells Dev* 19: 131-142.

Chougule P, Herlenius G, Hernandez NM, Patil PB, Xu B, Sumitran-Holgersson S (2012) Isolation and characterization of human primary enterocytes from small intestine using a novel method. *Scand J Gastroenterol* doi: 10.3109/00365521.2012.708940.

Church DM, Goodstadt L, Hillier LW, Zody MC, Goldstein S, She X, Bult CJ, Agarwala R, Cherry JL, DiCuccio M, Hlavina W, Kapustin Y, Meric P, Maglott D, Birtle Z, Marques AC, Graves T, Zhou S, Teague B, Potamousis K, Churas C, Place M, Herschleb J, Runnheim R, Forrest D, Amos-Landgraf J, Schwartz DC, Cheng Z, Lindblad-Toh K, Eichler EE, Ponting CP; Mouse Genome Sequencing Consortium (2009) Lineage-specific biology revealed by a finished genome assembly of the mouse. *PLoS Biol* 7: e1000112.

Claudianos C, Ranson H, Johnson RM, Biswas S, Schuler MA, Berenbaum MR, Feyereisen R, Oakeshott JG (2006) A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. *Insect Mol Biol* 15: 615-636.

Crespi CL, Penman BW, Hu M (1996) Development of Caco-2 cells expressing high levels of cDNA-derived cytochrome P4503A4. *Pharm Res* 13: 1635-1641.

Crow JA, Borazjani A, Potter PM, Ross MK (2007) Hydrolysis of pyrethroids by human and rat tissues: examination of intestinal, liver and serum carboxylesterases. *Toxicol Appl Pharmacol* 221: 1-12.

de Jong FA, Scott-Horton TJ, Kroetz DL, McLeod HL, Friberg LE, Mathijssen RH, Verweij J, Marsh S, Sparreboom A (2007) Irinotecan-induced diarrhea; functional significance of the polymorphic ABCC2 transporter protein. *Clin Pharmacol Ther* 81: 42-49.

Des Rieux A, Fievez V, Théate I, Mast J, Préat V, Schneider YJ (2007) An improved *in vitro* model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells. *Eur J Pharm Sci* 30: 380-391.

Dickson M, Gagnon JP (2004) Key factors in the rising cost of new drug discovery and development. *Nat Rev Drug Discov* 3: 417-429.

DiMasi JA, Hansen RW, Grabowski HG (2003) The price of innovation: new estimates of drug development costs. *J Health Econ* 22: 151-185.

Directive 2010/63/EU, OJ L 276, p. 33 to 79 of 20.10.2010

Directive number 63 of 2010, *Official Journal* of 20 October 2010, L 276, pages 33 to 79.

Dubreuil JD (2012) The whole Shebang: the gastrointestinal tract, *Escherichia coli* enterotoxins and secretion. *Curr Issues Mol Biol* 14: 71-82.

Durand N, Chertemps T, Maïbèche-Coisne M (2012) Antennal carboxylesterases in a moth, structural and functional diversity. *Commun Integr Biol* 5: 284-286.

Elamin E, Jonkers D, Juuti-Uusitalo K, can Ijzendoorn S, Troost F, Duimel H, Broers J, Verheyen F, Dekker J, Masclee A (2012) Effects of ethanol and acetaldehyde on tight junction integrity. *In vitro* study in a three dimensional intestinal epithelial cell culture model. *PLoS One* 7: e35008.

Engle MJ, Goetz GS, Alpers DH (1998) Caco-2 cells express a combination of colonocyte and enterocyte phenotypes *J Cell Physiol* 174: 362-369.

European Medicines Agency. Guideline on the Investigation of Drug Interactions. EMA/CHMP/EWP/125211/2010 (European Medicines Agency, London, UK, 2012). http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/05/WC500090112.pdf (2012).

Fagerholm U (2007) Prediction of human pharmacokinetics – gastrointestinal absorption. *J Pharm Pharmacol* 59: 905-916.

Ferruzza S, Rossi C, Scarino ML, Sambuy Y (2012) A protocol for differentiation of human intestinal Caco-2 cells in asymmetric serum-containing medium. *Toxicol In Vitro* doi: 10-1016/j.tiv.2012.01.008.

Fleet JC (2007) Using genomics to understand intestinal biology. *J Physiol Biochem* 63: 83-96.

Fogh J, Fogh JM, Orfeo T (1977) One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 59: 221-226.

Food and Drug Administration. FDA Guidance for industry, waiver of *in vivo* bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system. (Food and Drug Administration, Baltimore, MD, 2000). http://www.fda.gov/cder/guidance/3618fnl.htm (2012).

Fukami T, Yokoi T (2012) The Emerging Role of Human Esterases. *Drug Metab Pharmacokinet* doi: 10.2133/dmpk.DMPK-12-RV-042.

Furihata T, Hosokawa M, Nakata F, Satoh T, Chiba K (2003) Purification, molecular cloning, and functional expression of inducible liver acylcarnitine hydrolase in C57BL/6 mouse, belonging to the carboxylesterase multigene family. *Arch Biochem Biophys* 416: 101-109.

Geerts T, Vander Heyden T (2011) *In silico* predictions of ADME-Tox properties: drug absorption. *Comb Chem High Throughput Screen* 14: 339-361.

Gibbs RA, Weinstock GM, Metzker ML, Muzny DM, Sodergren EJ, Scherer S, Scott G, Steffen D, Worley KC, Burch PE, Okwuonu G, Hines S, Lewis L, DeRamo C, Delgado O, Dugan-Rocha S, Miner G, Morgan M, Hawes A, Gill R, Celera, Holt RA, Adams MD, Amanatides PG, Baden-Tillson H, Barnstead M, Chin S, Evans CA, Ferriera S, Fosler C, Glodek A, Gu Z, Jennings D, Kraft CL, Nguyen

T, Pfannkoch CM, Sitter C, Sutton GG, Venter JC, Woodage T, Smith D, Lee HM, Gustafson E, Cahill P, Kana A, Doucette-Stamm L, Weinstock K, Fechtel K, Weiss RB, Dunn DM, Green ED, Blakesley RW, Bouffard GG, De Jong PJ, Osoegawa K, Zhu B, Marra M, Schein J, Bosdet I, Fjell C, Jones S, Krzywinski M, Mathewson C, Siddiqui A, Wye N, McPherson J, Zhao S, Fraser CM, Shetty J, Shatsman S, Geer K, Chen Y, Abramzon S, Nierman WC, Havlak PH, Chen R, Durbin KJ, Egan A, Ren Y, Song XZ, Li B, Liu Y, Qin X, Cawley S, Worley KC, Cooney AJ, D'Souza LM, Martin K, Wu JQ, Gonzalez-Garay ML, Jackson AR, Kalafus KJ, McLeod MP, Milosavljevic A, Virk D, Volkov A, Wheeler DA, Zhang Z, Bailey JA, Eichler EE, Tuzun E, Birney E, Mongin E, Ureta-Vidal A, Woodwark C, Zdobnov E, Bork P, Suyama M, Torrents D, Alexandersson M, Trask BJ, Young JM, Huang H, Wang H, Xing H, Daniels S, Gietzen D, Schmidt J, Stevens K, Vitt U, Wingrove J, Camara F, Mar Albà M, Abril JF, Guigo R, Smit A, Dubchak I, Rubin EM, Couronne O, Poliakov A, Hübner N, Ganten D, Goesele C, Hummel O, Kreitler T, Lee YA, Monti J, Schulz H, Zimdahl H, Himmelbauer H, Lehrach H, Jacob HJ, Bromberg S, Gullings-Handley J, Jensen-Seaman MI, Kwitek AE, Lazar J, Pasko D, Tonellato PJ, Twigger S, Ponting CP, Duarte JM, Rice S, Goodstadt L, Beatson SA, Emes RD, Winter EE, Webber C, Brandt P, Nyakatura G, Adetobi M, Chiaromonte F, Elnitski L, Eswara P, Hardison RC, Hou M, Kolbe D, Makova K, Miller W, Nekrutenko A, Riemer C, Schwartz S, Taylor J, Yang S, Zhang Y, Lindpaintner K, Andrews TD, Caccamo M, Clamp M, Clarke L, Curwen V, Durbin R, Eyras E, Searle SM, Cooper GM, Batzoglou S, Brudno M, Sidow A, Stone EA, Venter JC, Payseur BA, Bourque G, López-Otín C, Puente XS, Chakrabarti K, Chatterji S, Dewey C, Pachter L, Bray N, Yap VB, Caspi A, Tesler G, Pevzner PA, Haussler D, Roskin KM, Baertsch R, Clawson H, Furey TS, Hinrichs AS, Karolchik D, Kent WJ, Rosenbloom KR, Trumbower H, Weirauch M, Cooper DN, Stenson PD, Ma B, Brent M, Arumugam M, Shteynberg D, Copley RR, Taylor MS, Riethman H, Mudunuri U, Peterson J, Guyer M, Felsenfeld A, Old S, Mockrin S, Collins F; Rat Genome Sequencing Project Consortium (2004) Genome sequence of the Brown Norway rat yields insights into mammalian evolution. *Nature* 428: 493-521.

Gumbiner BM (1996) Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 84: 345-357.

Hakamata W, Machida A, Oku T, Nishio T (2011) Design and synthesis of an ER-specific fluorescent probe based on carboxylesterase activity with quinone methide cleavage process. *Bioorg Med Chem Lett* 21: 3206-3209.

Harrison AP, Erlwanger KH, Elbrønd VS, Andersen NK, Unmack MA (2004) Gastrointestinal-tract model and techniques for use in safety pharmacology. *J Pharmacol Toxicol Methods* 49: 187-199.

Hatfield MJ, Tsurkan L, Hyatt JL, Yu X, Edwards CC, Hicks LD, Wadkins RM, Potter PM (2010) Biochemical and molecular analysis of carboxylesterase-mediated hydrolysis of cocaine and heroin. *Br J Pharmacol* 160: 1916-1928.

Hatfield MJ, Tsurkan L, Garrett M, Shaver TM, Hyatt JL, Edwards CC, Hicks LD, Potter PM (2011) Organ-specific carboxylesterase profiling identifies the small intestine and kidney as major contributors of activation of the anticancer prodrug CPT-11. *Biochem Pharmacol* 81: 24-31.

Hayeshi R, Hilgendorf C, Artursson P, Augustijns P, Brodin B, Dehertogh P, Fisher K, Fossati L, Hovenkamp E, Korjamo T, Masungi C, Maubon N, Mols R, Müllertz A, Mönkkönen J, O'Driscoll C, Oppers-Tiemissen HM, Ragnarsson EG, Rooseboom M, Ungell AL (2008) Comparison of drug transporter gene expression and functionality in Caco-2 cells from 10 different laboratories. *Eur J Pharm Sci* 35: 383-396.

Heikkinen AT, Baneyx G, Caruso A, Parrot N (2012) Application of PBPK modelling to predict human intestinal metabolism of CYP3A substrates – An evaluation and case study using GastroPlus™. *Eur J Pharm Sci* 47: 375-386.

Hidalgo IJ, Raub TJ, Borchardt RT (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal permeability. *Gastroenterology* 96: 736-749.

Holmes RS, Chan J, Cox LA, Murphy WJ, VandeBerg JL (2008) Opossum carboxylesterases: sequences, phylogeny and evidence for *CES* gene duplication events predating the marsupial-eutherian common ancestor. *BMC Evol Biol* 8: 54.

Holmes RS, Glenn JP, VandeBerg JL, Cox LA (2009) Baboon carboxylesterases 1 and 2: sequences, structures and phylogenetic relationships with human and other primate carboxylesterases. *J Med Primatol* 38: 27-38.

Holmes RS, Wright MW, Laulederkind SJ, Cox LA, Hosokawa M, Imai T, Ishibashi S, Lehner R, Miyazaki M, Perkins EJ, Potter PM, Redinbo MR, Robert J, Satoh T, Yamashita T, Yan B, Yokoi T, Zechner R, Maltais LJ (2010) Recommended nomenclature for five mammalian carboxylesterase gene families: human, mouse, and rat genes and proteins. *Mamm Genome* 21: 427-441.

Hotelier T, Renault L, Cousin X, Negre V, Marchot P, Chatonnet A (2004) ESTHER, the database of the alpha/beta-hydrolase fold superfamily of proteins. *Nucleic Acid Res* 32: D145-147.

Hubatsch I, Ragnarsson EG, Artursson P (2007) Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat Protoc* 2: 2111-2119.

Hull RN, Cherry WR, Weaver GW (1976) The origin and characteristics of a pig kidney cell strain, LLC-PK. *In Vitro* 12: 670-677.

Humerickhouse R, Lohrbach K, Li L, Bosron WF, Dolan ME (2000) Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. *Cancer Res* 60: 1189-1192.

Huynh L, Masereeuw R, Friedberg T, Ingelman-Sundberg M, Manivet P (2009) *In silico* platform for xenobiotics ADME-T pharmacological properties modelling and prediction. Part I: Beyond the reduction of animal model use. *Drug Discov Today* 14: 401-405.

Innocenti F, Kroetz DL, Schuetz E, Dolan ME, Ramírez J, Relling M, Chen P, Das S, Rosner GL, Ratain MJ (2009) Comprehensive pharmacogenetic analysis of irinotecan neutropenia and pharmacokinetics. *J Clin Oncol* 27: 2604-2614.

Irvine JD, Takahashi L, Lockhart K, Cheong J, Tolan JW, Selick HE, Grove JR (1999) MDCK (Madin-Darby canine kidney) cells: A tool for membrane permeability screening. *J Pharm Sci* 88: 28-33.

Jacob A, Pratuangdejkul J, Buffet S, Launay JM, Manivet P (2009) *In silico* platform for xenobiotics ADME-T pharmacological properties modelling and prediction. Part II: The body in a Hilbertian space. *Drug Discov Today* 14: 406-412.

Jeon JH, Kim SJ, Lee HS, Cha SS, Lee JH, Yoon SH, Koo BS, Lee CM, Choi SH, Lee SH, Kang SG, Lee JH (2011) Novel metagenome-derived carboxylesterase that hydrolyzes β-lactam antibiotics. *Appl Environm Microbiol* 77: 7830-7836.

Karpova MB, Schoumans J, Ernberg I, Henter JI, Nordenskjöld M, Fadeel B (2005) Raji revisited. Cytogenetics of the original Burkitt's lymphoma cell line. *Leukemia* 19: 159-161.

Kernéis S, Bogdanova A, Kraehenbuhl JP, Pringault E (1997) Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria. *Science* 277: 949-952.

Kim EB, Fang X, Fushan AA, Huang Z, Lobanov AV, Han L, Marino SM, Sun X, Turanov AA, Yang P, Yim SH, Zhao X, Kasaikina MV, Stoletzki N, Peng C, Polak P, Xiong Z, Kiezun A, Zhu Y, Chen Y, Kryukov GV, Zhang Q, Peshkin L, Yang L, Bronson RT, Buffenstein R, Wang B, Han C, Li Q, Chen L, Zhao W, Sunyaev SR, Park TJ, Zhang G, Wang J, Gladyshev VN (2011) Genome sequencing reveals insights into physiology and longevity of the naked mole rat. *Nature* 479: 223-227.

Kubo T, Kim SR, Sai K, Saito Y, Nakajima T, Matsumoto K, Saito H, Shirao K, Yamamoto N, Minami H, Ohtsu A, Yoshida T, Saijo N, Ohno Y, Ozawa S, Sawada J (2005) Functional characterization of three naturally occurring single nucleotide polymorphisms in the *CES2* gene encoding carboxylesterase 2 (HCE-2). *Drug Metab Dispos* 33: 1482-1487.

Kweekel D, Guchelaar HJ, Gelderblom H (2008) Clinical and pharmacogenetic factors associated with irinotecan toxicity. *Cancer Treat Rev* 34: 656-669.

Lagas JS, Damen CW, can Waterschoot RA, Iusuf D, Beijnen JH, Schinkel AH (2012) P-gp, Mrp2, Cyp3a, and carboxylesterase affect the oral availability and metabolism of vinorelbine. *Mol Pharmacol* doi: 10.1124/mol.111.077099.

Lee IJ, Hom K, Baj G, Shapiro M (2009) NMR metabolomics analysis of Caco-2 cell differentiation. *J Proteome Res* 8: 4104-4108.

Lennernäs H (1998) Human intestinal permeability. *J Pharm Sci* 87: 403-410.

Li B, Sedlacek M, manoharan I, Boopathy R, Duysen EG, Masson P, Lockridge O (2005) Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. *Biochem Pharmacol* 70: 1673-1684.

Li J, Volpe DA, Wang Y, Zhang W, Bode C, Owen A, Idalgo IJ (2011) Use of transporter knockdown Caco-2 cells to investigate the *in vitro* efflux of statin drugs. *Drug Metab Dispos* 39: 1196-1202.

Li R, Fan W, Tian G, Zhu H, He L, Cai J, Huang Q, Cai Q, Li B, Bai Y, Zhang Z, Zhang Y, Wang W, Li J, Wei F, Li H, Jian M, Li J, Zhang Z, Nielsen R, Li D, Gu W, Yang Z, Xuan Z, Ryder OA, Leung FC, Zhou Y, Cao J, Sun X, Fu Y, Fang X, Guo X, Wang B, Hou R, Shen F, Mu B, Ni P, Lin R, Qian W, Wang G, Yu C, Nie W, Wang J, Wu Z, Liang H, Min J, Wu Q, Cheng S, Ruan J, Wang M, Shi Z, Wen M, Liu B, Ren X, Zheng H, Dong D, Cook K, Shan G, Zhang H, Kosiol C, Xie X, Lu Z, Zheng H, Li Y, Steiner CC, Lam TT, Lin S, Zhang Q, Li G, Tian J, Gong T, Liu H, Zhang D, Fang L, Ye C, Zhang J, Hu W, Xu A, Ren Y, Zhang G, Bruford MW, Li Q, Ma L, Guo Y, An N, Hu Y, Zheng Y, Shi Y, Li Z, Liu Q, Chen Y, Zhao J, Qu N, Zhao S, Tian F, Wang X, Wang H, Xu L, Liu X, Vinar T, Wang Y, Lam TW, Yiu SM, Liu S, Zhang H, Li D, Huang Y, Wang X, Yang G, Jiang Z, Wang J, Qin N, Li L, Li J, Bolund L, Kristiansen K, Wong GK, Olson M, Zhang X, Li S, Yang H, Wang J, Wang J (2010) The sequence and de novo assembly of the giant panda genome. *Nature* 463: 311-317.

Liederer BM, Borchardt RT (2006) Enzymes involved in the bioconversion of ester-based prodrugs. *J Pharm Sci* 95: 1177-1195.

Lin JH, Chiba M, Baillie TA (1999) Is the role of the small intestine in first-pass metabolism overemphasized? *Pharmacol Rev* 51: 135-158.

Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, Clamp M, Chang JL, Kulbokas EJ 3rd, Zody MC, Mauceli E, Xie X, Breen M, Wayne RK, Ostrander EA, Ponting CP, Galibert F, Smith DR, DeJong PJ, Kirkness E, Alvarez P, Biagi T, Brockman W, Butler J, Chin CW, Cook A, Cuff J, Daly MJ, DeCaprio D, Gnerre S, Grabherr M, Kellis M, Kleber M, Bardeleben C, Goodstadt L, Heger A, Hitte C, Kim L, Koepfli KP, Parker HG, Pollinger JP, Searle SM, Sutter NB, Thomas R, Webber C, Baldwin J, Abebe A, Abouelleil A, Aftuck L, Ait-Zahra M, Aldredge T, Allen N, An P, Anderson S, Antoine C, Arachchi H, Aslam A, Ayotte L, Bachantsang P, Barry A, Bayul T, Benamara M, Berlin A, Bessette D, Blitshteyn B, Bloom T, Blye J, Boguslavskiy L, Bonnet C, Boukhgalter B, Brown A, Cahill P, Calixte N, Camarata J, Cheshatsang Y, Chu J, Citroen M, Collymore A, Cooke P, Dawoe T, Daza R, Decktor K, DeGray S, Dhargay N, Dooley K, Dooley K, Dorje P, Dorjee K, Dorris L, Duffey N, Dupes A, Egbiremolen O, Elong R, Falk J, Farina A, Faro S, Ferguson D, Ferreira P, Fisher S, FitzGerald M, Foley K, Foley C, Franke A, Friedrich D, Gage D, Garber M, Gearin G, Giannoukos G, Goode T, Goyette A, Graham J, Grandbois E, Gyaltsen K, Hafez N, Hagopian D, Hagos B, Hall J, Healy C, Hegarty R, Honan T, Horn A, Houde N, Hughes L, Hunnicutt L, Husby M, Jester B, Jones C, Kamat A, Kanga B, Kells C, Khazanovich D, Kieu AC, Kisner P, Kumar M, Lance K, Landers T, Lara M, Lee W, Leger JP, Lennon N, Leuper L, LeVine S, Liu J, Liu X, Lokyitsang Y, Lokyitsang T, Lui A, Macdonald J, Major J, Marabella R, Maru K, Matthews C, McDonough S, Mehta T, Meldrim J, Melnikov A, Meneus L, Mihalev A, Mihova T, Miller K, Mittelman R, Mlenga V, Mulrain L, Munson G, Navidi A, Naylor J, Nguyen T, Nguyen N, Nguyen C, Nguyen T, Nicol R, Norbu N, Norbu C, Novod N, Nyima T, Olandt P, O'Neill B, O'Neill K, Osman S, Oyono L, Patti C, Perrin D, Phunkhang P, Pierre F, Priest M, Rachupka A, Raghuraman S, Rameau R, Ray V, Raymond C, Rege F, Rise C, Rogers J, Rogov P, Sahalie J, Settipalli S, Sharpe T, Shea T, Sheehan M, Sherpa N, Shi J, Shih D, Sloan J, Smith C, Sparrow T, Stalker J, Stange-Thomann N, Stavropoulos S, Stone C, Stone S, Sykes S, Tchuinga P, Tenzing P, Tesfaye S, Thoulutsang D, Thoulutsang Y, Topham K, Topping I, Tsamla T, Vassiliev H, Venkataraman V, Vo A, Wangchuk T, Wangdi T, Weiand M, Wilkinson J, Wilson A, Yadav S, Yang S, Yang X, Young G, Yu Q, Zainoun J, Zembek L, Zimmer A, Lander ES (2005) Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* 438: 803-819.

Linnankoski J, Mäkelä J, Pamgren J, Mauriala T, Vedin C, Ungell AL, Lazorova L, Artursson P, Urtti A, Yliperttula M (2010) Paracellular porosity and pore size of the human intestinal epithelium in tissue and cell culture models. *J Pharm Sci* 99: 2166-2175.

Marsh S, Hoskins JM (2010) Irinotecan pharmacogenomics. *Pharmacogenomics* 11: 1003-1010.

Marsh S, Xiao M, Yu J, Ahluwalia R, Minton M, Freimuth RR, Kwok PY, McLeod HL (2004) Pharmacogenomic assessment of carboxylesterases 1 and 2. *Genomics* 84: 661-668.

Melcher R, Koehler S, Steinlein C, Schmid M, Mueller CR, Luehrs H, Menzel T, Scheppach W, Moerk H, Scheurlen M, Koehrle J, Al-Taie O (2002) Spectral karyotype analysis of colon cancer cell lines of the tumor suppressor and mutator pathway. *Cytogenet Genome Res* 98: 22-28.

Morgan EW, Yan B, Greenway D, Parkinson A (1994) Regulation of two rat liver microsomal carboxylesterase isozymes: species differences, tissue distribution, and the effects of age, sex and xenobiotic treatment of rats. *Arch Biochem Biophys* 315: 513-526.

Mural RJ, Adams MD, Myers EW, Smith HO, Miklos GL, Wides R, Halpern A, Li PW, Sutton GG, Nadeau J, Salzberg SL, Holt RA, Kodira CD, Lu F, Chen L, Deng Z, Evangelista CC, Gan W, Heiman TJ, Li J, Li Z, Merkulov GV, Milshina NV, Naik AK, Qi R, Shue BC, Wang A, Wang J, Wang X, Yan X, Ye J, Yooseph S, Zhao Q, Zheng L, Zhu SC, Biddick K, Bolanos R, Delcher AL, Dew IM, Fasulo D, Flanigan MJ, Huson DH, Kravitz SA, Miller JR, Mobarry CM, Reinert K, Remington KA, Zhang Q, Zheng XH, Nusskern DR, Lai Z, Lei Y, Zhong W, Yao A, Guan P, Ji RR, Gu Z, Wang ZY, Zhong F, Xiao C, Chiang CC, Yandell M, Wortman JR, Amanatides PG, Hladun SL, Pratts EC, Johnson JE, Dodson KL, Woodford KJ, Evans CA, Gropman B, Rusch DB, Venter E, Wang M, Smith TJ, Houck JT, Tompkins DE, Haynes C, Jacob D, Chin SH, Allen DR, Dahlke CE, Sanders R, Li K, Liu X, Levitsky AA, Majoros WH, Chen Q, Xia AC, Lopez JR, Donnelly MT, Newman MH, Glodek A, Kraft CL, Nodell M, Ali F, An HJ, Baldwin-Pitts D, Beeson KY, Cai S, Carnes M, Carver A, Caulk PM, Center A, Chen YH, Cheng ML, Coyne MD, Crowder M, Danaher S, Davenport LB, Desilets R, Dietz SM, Doup L, Dullaghan P, Ferriera S, Fosler CR, Gire HC, Gluecksmann A, Gocayne JD, Gray J, Hart B, Haynes J, Hoover J, Howland T, Ibegwam C, Jalali M, Johns D, Kline L, Ma DS, MacCawley S, Magoon A, Mann F, May D, McIntosh TC, Mehta S, Moy L, Moy MC, Murphy BJ, Murphy SD, Nelson KA, Nuri Z, Parker KA, Prudhomme AC, Puri VN, Qureshi H, Raley JC, Reardon MS, Regier MA, Rogers YH, Romblad DL, Schutz J, Scott JL, Scott R, Sitter CD, Smallwood M, Sprague AC, Stewart E, Strong RV, Suh E, Sylvester K, Thomas R, Tint NN, Tsonis C, Wang G, Wang G, Williams MS, Williams SM, Windsor SM, Wolfe K, Wu MM, Zaveri J, Chaturvedi K, Gabrielian AE, Ke Z, Sun J, Subramanian G, Venter JC, Pfannkoch CM, Barnstead M, Stephenson LD (2002) A comparison of whole-genome shotgun-derived mouse chromosome 16 and the human genome. *Science* 296: 1661-1771.

Natoli M, Leoni BD, D'Agnano I, D'Onofrio M, Brandi R, Arisi I, Zucco F, Felsani A (2011) Cell growing density affects the structural and functional properties of Caco-2 differentiated monolayer. *J Cell Physiol* 226: 1531-1543.

Natoli M, Leoni BD, D'Agnano I, Zucco F, Felsani A (2012) Good Caco-2 cell culture practices. *Toxicol In Vitro* doi: 10.1016/j.tiv.2012.03.009.

Nicoletti C (2000) Unsolved mysteries of intestinal M cells. *Gut* 47: 735-739.

Nishimura M, Naito S (2006) Tissue-specific mRNA expression profiles of human phase I metabolizing enzymes except for cytochrome P450 and phase II metabolizing enzymes. *Drug Metab Pharmacokinet* 21: 357-374.

Ohura K, Sakamoto H, Ninomiya S, Imai T (2010) Development of a novel system for estimating human intestinal absorption using Caco-2 cells in the absence of esterase activity. *Drug Metab Dispos* 38: 323-331.

Ozols J (1989) Isolation, properties, and the complete amino acid sequence of a second form of 60-kDa glycoprotein esterase. Orientation of the 60-kDa proteins in the microsomal membrane. *J Biol Chem* 264: 12533-12545.

Paul SM, Mytelka DS, Dunwiddie CT, Persinger CC, Munos BH, Lindborg SR, Schacht AL (2010) How to improve R&D productivity: the pharmaceutical industry's grand challenge. *Nat Rev Drug Discov* 9: 203-214.

Perreault N, Beaulieu JF (1998) Primary cultures of fully differentiated and pure intestinal epithelial cells. *Exp Cell Res* 245: 34-42.

Pindel EV, Kedishvili NY, Abraham TL, Brzezinski MR, Zhang J, Dean RA, Bosron WF (1997) Purification and cloning of a broad substrate specificity human liver carboxylesterase that catalyzes the hydrolysis of cocaine and heroin. *J Biol Chem* 272: 14769-14775.

Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simon-Assmann P, Haffen K, Fogh J, Zweibaum A (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* 47: 323-330.

Prieto P, Hoffmann S, Tirelli V, Tancredi F, González I, Bermejo M, De Angelis I (2010) An exploratory study of two Caco-2 cell models for oral absorption: a report on their within-laboratory and between-laboratory variability, and their predictive capacity. *Altern Lab Anim* 38: 367-386.

Prieto P, Kinsner-Ovaskainen A, Stanzel S, Albella B, Artursson P, Campillo N, Cecchelli R, Cerrato L, Díaz L, Di Consiglio E, Guerra A, Gombau L, Herrera G, Honegger P, Landry C, O'Connor JE, Páez JA, Quintas G, Svensson R, Turco L, Zurich MG, Zurbano MJ, Kopp-Schneider A (2012) The value of selected in vitro and in silico methods to predict acute oral toxicity in a regulatory context: Results from the European Project ACuteTox. *Toxicol In Vitro* doi.org/10.1016/j.tiv.2012.07.013

Pulvertaft RJV (1964) Cytology of Burkitt's tumour (African lymphoma) *Lancet* 283: 238-240.

Ramond MJ, Martinot-Peignoux M, Erlinger S (1985) Dome formation in the huma colon carcinoma cell line Caco-2 in culture. Influence of oubain and permeable supports. *Biol Cell* 54: 89-92.

Randall KJ, Turton J, Foster JR (2011) Explant culture of gastrointestinal tissue: a review of methods and application. *Cell Biol Toxicol* 27: 267-284.

Redinbo MR, Potter PM (2005) Mammalian carboxylesterases: from drug targets to protein therapeutics. *Drug Discov Today* 10: 313-325.

Rejón JD, Zienkiewicz A, Rodríguez-García MI, Castro AJ (2012) Profiling and functional classification of esterases in olive (*Olea europaea*) pollen during germination. *Ann Bot* doi: 10.1093/aob/mcs174.

Renault L, Nègre V, Hotelier T, Cousin X, Marchot P, Chatonnet A (2005) New friendly tools for users of ESTHER, the database of the alpha/beta-hydrolase fold superfamily of proteins. *Chem Biol Interact* 157-158: 339-343.

Rizk P, Barker N (2012) Gut stem cells in tissue renewal and disease: methods, markers, and myths. *Wiley Interdiscip Rev Syst Biol Med* 4: 475-496.

Rodolosse A, Barbat A, Chantret I, Lacasa M, Brot-Laroche E, Zweibaum A, Rousset M (1998) Selecting agent hygromycin B alters expression of glucose-regulated genes in transfected Caco-2 cells. *Am J Physiol* 274: G931-G938.

Romero-Calvo I, Mascaraque C, Zarzuelo A, Suárez MD, martínez-Augustin O, de Medina FS (2011) Intestinal inflammation and the enterocyte transportome. *Biochem Soc Trans* 39: 1096-1101.

Ross MK, Borazjani A, Wang R, Crow JA, Xie S (2012) Examination of the carboxylesterase phenotype in human liver. *Arch Biochem Biophys* 522: 44-56.

Ross MK, Crow JA (2007) Human carboxylesterases and their role in xenobiotic and endobiotic metabolism. *J Biochem Mol Toxicol* 21: 187-196.

Rossi C, Guantario B, Ferruzza S, Guguen-Guillouzo C, Sambuy Y, Scarino ML, Bellovino D (2012) Co-cultures of enterocytes and hepatocytes for retinoid transport and metabolism. *Toxicol In Vitro* doi: 10.106/j.tiv.2012.04.013.

Roth WJ, Lindley DJ, Carl SM, Knipp GT (2012) The effects of intralaboratory modifications to media composition and cell source on the expression of pharmaceutically relevant transporters and metabolizing genes in the Caco-2 cell line. *J Pharm Sci* 101: 3962-3978.

Rousset M (1986) The human colon carcinoma cell lines HT-239 and Caco-2: two *in vitro* models for the study of intestinal differentiation. *Biochimie* 68: 1035-1040.

Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F (2005) The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol* 21: 1-26.

Santos A, Zanetta S, Cresteil T, Deroussent A, Pein F, Raymond E, Vernillet L, Risse ML, Boige V, Gouyette A, Vassal G (2000) Metabolism of irinotecan (CPT-11) by CYP3A4 and CYP3A5 in humans. *Clin Cancer Res* 6: 2012-2020.

Sato Y, Miyashita A, Iwatsubo T, Usui T (2012) Simultaneous absolute protein quantification of carboxylesterase 1 and 2 in human liver tissue fractions using liquid chromatography-tandem mass spectrometry. *Drug Metab Dispos* 40: 1389-1396.

Satoh T, Hosokawa M (1998) The mammalian carboxylesterases: from molecules to functions. *Annu Rev Pharmacol Toxicol* 38: 257–288.

Satoh T, Hosokawa M (2006) Structure, function and regulation of carboxylesterases. *Chem Biol Interact* 162: 195-211.

Sanghani SP, Quinney SK, Fredenburg TB, Sun Z, Davis WI, Murry DJ, Cummings OW, Seitz DE, Bosron WF (2003) Carboxylesterases expressed in human colon tumor tissue and their role in CPT-11 hydrolysis. *Clin Cancer Res* 9: 4983-4991.

Sarmento B, Andrade F, da Silva SB, Rodrigues F, das Neves J, Ferreira D (2012) Cell-based *in vitro* models for predicting drug permeability. *Expert Opin Drug Metab Toxicol* 8: 607-621.

Schiel MA, Green SL, Davis WI, Sanghani PC, Bosron WF, Sanghani SP (2007) Expression and characterization of a human carboxylesterase 2 splice variant. *J Pharmacol Exp Ther* 323: 94-101.

Schiffelers MJ, Blaauboer BJ, Hendriksen CF, Bakker WE (2012) Regulatory acceptance and use of 3R models: a multilevel perspective. *ALTEX* 29: 287-300.

Schmiedlin-Ren P, Thummel KE, Fisher JM, Paine MF, Watkins PB (2001) Induction of CYP3A4 by 1 alpha,25-dihydroxyvitamin D3 is human cell line-specific and is unlikely to involve pregnane X receptor. *Drug Metab Dispos* 29: 1446-1453.

Schroeder K, Bremm KD, Alépée N, Bessems JG, Blaauboer B, Boehn SN, Burek C, Coecke S, Gombau L, Hewitt NJ, Heylings J, Huwyler J, Jaeger M, Jagelavicius M, Jarrett N, Ketelslegers H, Kocina I, Koester J, Kreysa J, Note R, Poth A, Radtke M, Rogiers V, Scheel J, Schulz T, Steikellner H, Toeroek M, Winkler P, Diembeck W (2011) Report from the EPAA workshop: *in vitro* ADME in safety testing used by EPAA industry sectors. *Toxicol In Vitro* 25: 589-604.

Scripture CD, Figg WD (2006) Drug interactions in cancer therapy. *Nat Rev Cancer* 6: 546-558.

Shen DD, Kunze KL, Thummel KE (1997) Enzyme-catalyzed processes of first-pass hepatic and intestinal drug extraction. *Adv Drug Deliv Rev* 27: 99-127.

Siissalo S, Laitinen L, Koljonen M, Vellonen KS, Kortejärvi H, Urtti A, Hirvonen J, Kaukonen AM (2007) Effect of cell differentiation and passage number on the expression of efflux proteins in wild type and vinblastine-induced Caco-2 cell lines. *Eur J Pharm Biopharm* 67: 548-554.

Simon-Assmann P, Turck N, Sidhoum-Jenny M, Gradwohl G, Kedinger M (2007) *In vitro* models of intestinal epithelial cell differentiation. *Cell Biol Toxicol* 23: 241-256.

Sone T, Isobe M, Takabatake E, Wang CY (1994) Cloning and sequence analysis of a hamster liver cDNA encoding a novel putative carboxylesterase. *Biochim Biophys Acta* 1207: 138-142.

Stegmann A, Hansen M, Wang Y, Larsen JB, Lund LR, Ritié L, Nicholson JK, Quistorff B, Simon-Assmann P, Troelsen JT, Olsen J (2006) Metabolome, transcriptome, and bioinformatics cis-element analyses point to HNF-4 as a central regulator of gene expression during enterocyte differentiation. *Physiol Genomics* 27: 141-155.

Tabata T, Katoh M, Tokudome S, Nakajima M, Yokoi T (2004) Identification of the cytosolic carboxylesterase catalyzing the 5'-deoxy-5-fluorocytidine formation from capecitabine in human liver. *Drug Metab Dispos* 10: 1103-1110.

Tang M, Mukundan M, Yang J, Charpentier N, LeCluyse EL, Bkack C, Yang D, Shi D, Yan B (2006) Antiplatelet agents aspirin and clopidogrel are hydrolysed by distinct carboxylesterases, and clopidogrel is transesterificated in the presence of ethyl alcohol. *J Pharmacol Exp Ther* 319: 1467-1476.

Tartar A, Wheeler MM, Zhou X, Coy MR, Boucias DG, Scharf ME (2009) Parallel metatranscriptome analyses of host and symbiont gene expression in the gut of the termite *Reticulitermes flavipes*. *Biotechnol Biofuels* 2: 35.

Tavelin S, Milovic V, Oklind G, Olsson S, Artursson P (1999) A conditionally immortalized epithelial cell line for studies of intestinal drug transport. *J Pharmacol Exp Ther* 290: 1212-1221.

Theil FP, Guentert TW, Haddad S, Poulin P (2003) Utility of physiologically based pharmacokinetic models to drug development and rational drug discovery candidate selection. *Toxicol Lett* 138: 29-49.

Thelen K, Dressman JB (2009) Cytochrome P450-mediated metabolism in the human gut wall. *Pharm Pharmacol* 61: 541-558.

Thorn CF, Carrillo MW, Ramirez J, Gong L, Marsh S, Schuetz EG, Dolan ME, Innocenti F, Relling MV, McLeod HL, Ratain MJ (2003) Irinotecan pathway. *PharmGKB*: available online http://www.pharmgkb.org/pathway/PA2001#PGG.

Tsurkan LG, Hatfield MJ, Edwards CC, Hyatt JL, Potter PM (2012) Inhibition of human carboxylesterases hCE1 and hiCE by cholinesterase inhibitors. *Chem Biol Interact* doi: 10.1016/j.cbi.2012.10.018

Uchino J, Takayama K, Harada A, Sone T, harada T, Curiel DT, Kuroki M, Nakanishi Y (2008) Tumor targeting carboxylesterase fused with anti-CEA scFv improve the anticancer effect with a less toxic dose of irinotecan. *Cancer Gene Ther* 15: 94-100.

Ungell AL (2004) Caco-2 replace or refine? *Drug Discov Today Technol* 4: 423-430.

van de Waterbeemd H, Gifford E (2003) ADMET *in silico* modelling: towards prediction paradise? *Nat Rev Drug Discov* 2: 192-204.

van Waterschoot RA, Schinkel AH (2011) A critical analysis of the interplay between cytochrome P450 3A and P-glycoprotein: recent insights from knockout and transgenic mice. *Pharmacol Rev* 63: 390-410.

Vereecke L, Beyaert R, van Loo G (2011) Enterocyte death and intestinal barrier maintenance in homeostasis and disease. *Trends Mol Med* 17: 584-593.

Volpe DA (2010) Application of method suitability for drug permeability classification. *AAPS J* 12: 670-678.

Wade CM, Giulotto E, Sigurdsson S, Zoli M, Gnerre S, Imsland F, Lear TL, Adelson DL, Bailey E, Bellone RR, Blöcker H, Distl O, Edgar RC, Garber M, Leeb T, Mauceli E, MacLeod JN, Penedo MC, Raison JM, Sharpe T, Vogel J, Andersson L, Antczak DF, Biagi T, Binns MM, Chowdhary BP, Coleman SJ, Della Valle G, Fryc S, Guérin G, Hasegawa T, Hill EW, Jurka J, Kiialainen A, Lindgren G, Liu J, Magnani E, Mickelson JR, Murray J, Nergadze SG, Onofrio R, Pedroni S, Piras MF, Raudsepp T, Rocchi M, Røed KH, Ryder OA, Searle S, Skow L, Swinburne JE, Syvänen AC, Tozaki T, Valberg SJ, Vaudin M, White JR, Zody MC; Broad Institute Genome Sequencing Platform; Broad Institute Whole Genome Assembly Team, Lander ES, Lindblad-Toh K (2009) Genome sequence, comparative analysis, and population genetics of the domestic horse. *Science* 326: 865-867.

Wang J, Williams ET, Bourgea J, Wong YN, Patten CJ (2011) Characterization of recombinant human carboxylesterases: fluorescein diacetate as a probe substrate for human carboxylesterase 2. *Drug Metab Dispos* 39: 1329-1333.

Wells DJ (2011) Animal welfare and the 3Rs in European biomedical research. *Ann N Y Acad Sci* 1245: 14-16.

Wheelock CE, Miller JL, Miller MJ, Phillips BM, Huntley SA, Gee SJ, Tjeerdema RS, Hammock BD (2006) Use of carboxylesterase activity to remove pyrethroid-associated toxicity to *Ceriodaphnia dubia* and *Hyalella Azteca* in toxicity identification evaluations. *Environ Toxicol Chem* 25: 973-984.

Williams ET, Wang H, Wrighton SA, Qian YW, Perkins EJ (2010) Genomic analysis of the carboxylesterases: identification and classification of novel forms. *Mol Phylogenet Evol* 57: 23-34.

Williams ET, Bacon JA, Bender DM, Lowinger JJ, Guo WK, Ehsani ME, Wang X, Wang H, Qian YW, Ruterbories KJ, Wrighton SA, Perkins EJ (2011) Characterization of the expression and activity of carboxylesterases 1 and 2 from the beagle dog, cynomolgus monkey, and human. *Drug Metab Dispos* 39: 2305-2313.

Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, Peterkin V, Koup JR, Ball SE (2004) Drug-drug interations for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for tipically observed low exposure (AUCi/AUC) ratios. *Drug Metab Dispos* 32: 1201-1208.

Wu L, Martin TD, Carrington M, KewalRamani VN (2004b) Raji B cells, misidentified as THP-1 cells, stimulate DC-SIGN-mediated HIV transmission. *Virology* 318: 17-23.

Wu MH, Chen P, Remo BF, Cook EH Jr, Das S, Dolan ME (2003) Characterization of multiple promoters in the human carboxylesterase 2 gene. *Pharmacogenetics* 7: 425-435.

Wu MH, Chen P, Wu X, Liu W, Strom S, Das S, Cook EH Jr, Rosner GL, Dolan ME (2004) Determination and analysis of single nucleotide polymorphisms and haplotype structure of the human carboxylesterase 2 gene. *Pharmacogenetics* 14: 595-605.

Xu X, Nagarajan H, Lewis NE, Pan S, Cai Z, Liu X, Chen W, Xie M, Wang W, Hammond S, Andersen MR, Neff N, Passarelli B, Koh W, Fan HC, Wang J, Gui Y, Lee KH, Betenbaugh MJ, Quake SR, Famili I, Palsson BO, Wang J (2011) The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. *Nat Biotechnol* 29: 735-741.

Yang J, Shi D, Yang D, Song X, Yan B (2007) Interleukin-6 alters the cellular responsiveness to clopidogrel, irinotecan, and oseltamivir by suppressing the expression of carboxylesterases HCE1 and HCE2. *Mol Pharmacol* 72: 686-694.

Yano H, Kayukawa S, Iida S, Nakagawa C, Oguri T, Sanda T, Ding J, Mori F, Ito A, Ri M, Inagaki A, Kusumoto S, Ishida T, Komatsu H, Inagaki H, Suzuki A, Ueda R (2008) Overexpression of carboxylesterase-2 results in enhanced efficacy of topoisomerase I inhibitor, irinotecan (CPT-11), for multiple myeloma. *Cancer Sci* 99: 2309-2314.

Yoon KJ, Hyatt JL, Morton CL, Lee RE, Potter PM, Danks MK (2004) Characterization of inhibitors of specific carboxylesterases: development of carboxylesterase inhibitors for translational application. *Mol Cancer Ther.* 3: 903-909.

Zucco F, Batto AF, Bises G, Chambaz J, Chiusolo A, Consalvo R, Cross H, Dal Negro G, de Angelis I, Fabre G, Guillou F, Hoffman S, Laplanche L, Morel E, Pinçon-Raymond M, Prieto P, Turco L, Ranaldi G, Rousset M, Sambuy Y, Scarino ML, Torreilles F, Stammati A (2005) An inter-laboratory study to evaluate the effects of medium composition on the differentiation and barrier function of Caco-2 cell lines. *Alter Lab Anim* 33: 603-618.

SECTION 2

Analytical methodology development for CES2 activity evaluation

Adapted from:

Lamego J, Coroadinha AS, Simplício AL. (2011) Detection and quantification of carboxylesterase 2 activity by capillary electrophoresis. *Anal Chem* 83: 881-887.

and

Simplício AL, Coroadinha AS, Gilmer JF, **Lamego J**. A methodology for detection and quantification of esterase activity. *Capillary Electrophoresis of Biomolecules: Methods and Protocols*; in press.

This work has been partially presented, at the 2nd National Meeting on Medicinal Chemistry, held on the 28th of November of 2010 in Coimbra, Portugal.

Abstract

Carboxylesterases (CESs) are important enzymes for xenobiotic metabolism and are receiving increased attention in the context of cancer therapies. Quantification of individual CES activity is important since protein levels do not always correlate to activity and significant inter-organ, inter-individual, and interspecies variations exist.
The purpose of this study was to develop an analytical method to quantify the relative activities of CESs in biological samples. Taking advantage of loperamide, a selective carboxylesterase 2 (CES2) inhibitor, and bis-*p*-nitrophenyl phosphate (BNPP), an irreversible CESs inhibitor, a capillary electrophoresis (CE) method that enables detecting and distinguishing CES2 activity from other CESs in complex biological samples is proposed for the first time. The capillary electrophoresis method proved to be fast, simple, repeatable, and applicable to the measurement of the specific activity of CESs. The method was successfully applied to the evaluation of human cells overexpressing human CES2 (hCES2) and to several mammalian sera, using extremely small amounts of samples in comparison with traditional spectrophotometric methods. The same rationale can be applied to establish methods for determining the activity of other esterases, using the appropriate specific inhibitors or substrates.

Contents

2.1 Introduction

Mammalian carboxylesterases are α,β-hydrolase folded proteins involved in the efficient hydrolysis of xenobiotics and endogenous ester- or amide-containing compounds (Satoh and Hosokawa 1998; Satoh and Hosokawa 2006; Xie et al. 2002). The classification of these enzymes is difficult due to the large number of isoenzymes, complex tissue distribution, and overlapping substrate recognition (Satoh and Hosokawa 2006; Liederer and Borchardt 2006). The most recent classification of mammalian CESs categorises these enzymes into five subfamilies, from CES1 to CES5, according to their sequence homology (Satoh and Hosokawa 1998; Williams et al. 2010). Also, CES nomenclature was recently revisited, as explained in Section 1 (Holmes et al. 2010).

Carboxylesterases merit close attention because of their important functions in the detoxification of narcotics and other drugs, metabolism of pyrethroids, and prodrug activation (Hicks et al. 2009; Holmes et al. 2009). Several studies have been conducted to compare the enzymatic activities of the different CESs in mammals, due to the implications that these differences may have for (pre)clinical results (Xie et al. 2002; Li et al. 2005; Jewell et al. 2007a; Williams et al. 2011).

More recently, these enzymes have gained additional attention in the cancer treatment area. It has been discovered, for example, that the levels of some isoenzymes are different in certain tumours: human carboxylesterase 1 (hCES1) protein levels have been found to be increased in patients suffering from hepatocellular carcinoma (Na et al. 2009). Moreover, a correlation was established between hCES2 expression in solid tumours and the activation of the anticancer prodrug Irinotecan (CPT-11), and this has led to the investigation into enzyme/prodrug strategies with the goal to target expressed hCESs to tumours prior to the treatment with the prodrug (Xu G et al. 2002; Hatfield et al. 2008). Human carboxylesterase 2, the major human intestinal CES, thus attracts considerable

attention. It has been suggested that, prior to the treatment of human lung cancer cells with CPT-11, it is crucial to check if the cells express this enzyme (Satoh and Hosokawa 2006).

Considering the current trends, it becomes evident that fast, reliable, economical, and easy to perform analytical methods, enabling the evaluation of CES2 activity, will be needed for routine application in the near future. There are currently several ways to assess hCES2 expression levels and activities (Ross and Borazjani 2007), such as simple spectrophotometric (Crow et al. 2007), native in-gel hydrolysis (Ross and Crow 2007), Western blot (Kubo et al. 2005), real-time polymerase chain reaction (RT-PCR; Jewell et al. 2007a), and Northern blot assays (Zhu et al. 2000). A simple spectrophotometric assay enables one to quantify total CESs activity, but does not identify the relative activity of each specific CES in a complex biological sample or enzyme mixture since the majority of cases results in overlapping absorbance when inhibitors are used. A native in-gel hydrolysis assay allows one to distinguish the activities of the different CESs in complex biological samples, but only estimates on protein quantity are possible. With Western blot assays it is possible to detect specific proteins using appropriate antibodies, but only estimates concerning enzymatic activity or protein quantity are possible. RT-PCR and Northern blot assays enable one to quantify and detect, respectively the analysis of mRNA expression profiles; again, no information concerning enzyme activity is obtained (Ross and Borazjani 2007). As reported before, the protein levels of CESs do not always correlate with their activity levels (Ross and Crow 2007). As CESs are promiscuous enzymes with overlapping substrate recognition, it becomes difficult to attribute a specific activity to one isoenzyme in complex protein samples such as cellular extracts, serum, or other biological samples (Ross and Crow 2007; Adam et al. 2003). Accordingly, the classical substrate used for CES activity, *p*-nitrophenyl acetate (*p*-NPA), is also hydrolysed by cholinesterases, thus constituting an example of the substrate recognition of these enzymes. This overlapping substrate recognition often leads to contradictions in the

literature (Satoh and Hosokawa 2006); in 2005, it was clearly demonstrated that human healthy plasma does not contain CES activity (Li et al. 2005).

In this work, a new analytical methodology is provided for the CES field, which involves the use of several substrates and inhibitors enabling the identification and quantification of CES2 activity in complex biological samples. Capillary electrophoresis was chosen as the separation technique since simple spectrophotometric assessment is precluded due to overlapping absorbance between some substrates and inhibitors. Capillary electrophoresis is a powerful separation technique that has established itself as an alternative to chromatographic techniques (Elbashir et al. 2010). Capillary electrophoresis advantages rely on the reduced electrolyte and sample needs, the low cost of the capillaries, in comparison with the high-performance liquid chromatography (HPLC) columns, faster method development, and versatility (Fleury-Souverain et al. 2009). Nonetheless, HPLC can still be used with the proposed methodology. The method was successfully applied to the quantification of CES2 activity in transfected cell extracts and animal sera, and the same rationale can be quickly extended to the study of other enzymes and/or substrates in other biological samples.

2.2 Materials and Methods

2.2.1 Enzymes and reagents

Carboxylesterase 1 enzyme (esterase from porcine liver) was acquired from Sigma (Buchs, Switzerland); butyrylcholinesterase (BuChE) from equine serum and acetylcholinesterase (AcChE) from electric eel were also from Sigma (St. Louis, U.S.A.). Recombinant hCES2 was from R&D Systems, Inc. (Minneapolis, U.S.A.).

All reagents were analytical grade or higher. 4-Methylumbelliferyl acetate (4-MUBA) (≥98%), 4-methylumbelliferone (4-MUB) (≥98%), *p*-NPA (>99%), procaine hydrochloride (>97%), *p*-aminobenzoic acid (*p*-ABA) (>99%), Trizma hydrochloride

(>99%), and Trizma base (>99.9%) were from Sigma (St. Louis, U.S.A.). S-Butyrylthiocholine (BuTCh) iodide (≥99%) and acetylthiocholine (AcTCh) iodide (≥99%) were also from Sigma (Buchs, Switzerland). Loperamide hydrochloride (analytical grade) was from Fluka (Seelze, Germany), whereas bis-*p*-nitrophenyl phosphate (BNPP) (>99%) and potassium chloride (≥99.5%) were from Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate (≥99%), disodium tetraborate (≥99.5%), and ethanol (99.5%) were all from Panreac (Barcelona, Spain); dimethyl sulfoxide (DMSO) (95%) was from Lab-Scan (Dublin, Ireland), and sodium dihydrogen phosphate (>99%) was from Merck (Darmstadt, Germany).

2.2.2 Instrumentation

CE detection was performed using a Beckman Coulter (Palo Alto, U.S.A.) P/ACE MDQ capillary electrophoresis system equipped with a diode array detector. A fused silica capillary (Agilent; Santa Clara, U.S.A.) with 75 μm i.d., and 42 cm total length (32 cm to detector) was used.

The capillary was housed in a standard cartridge and maintained at 25 ^{o}C during all experiments. The sample tray was kept at 15 ^{o}C to minimize solution deterioration. Injections were conducted hydrodynamically, at 0.2 psi for 10 s, with cathodic migration. Separations were performed at constant current (+70.0 μA). UV absorption was measured at 230 (thiocholines), 254 (*p*-ABA), 280 (procaine), 350 (4-MUB) and 400 nm (*p*-nitrophenol (*p*-NP)).

Each new capillary was pretreated with sodium hydroxide 0.1 mM, rinsed with Milli-Q water, and conditioned with background electrolyte solution (BES), 30 mM borate-phosphate buffer (30 mM disodium tetraborate, 30 mM sodium dihydrogen phosphate; pH 8.0; Tang et al. 2007), at a pressure of 5.0 psi for 20 min each, for a total of 60 min. This procedure was repeated at the end of each day. The

capillary was conditioned between all runs, by a short sequence (30 s each) of this procedure.

Data acquisition, storage, and analysis were performed using P/ACE MDQ version 7.0. All the injected solutions used were diluted in equal proportion (50% *v/v*) of ethanol as the samples to be analysed.

2.2.3 Preparation of human cell extracts

Human embryonic kidney cells (HEK-293T; ATCC CRL-11268) were transfected as described before (Xie et al. 2002), following some modifications. In brief, cells were plated at $5x10^4$ cell.cm^{-2} and transfected, on the following day, at 60-80% of cell density with polycation polyethylenimine (PEI; Polysciences; Eppelheim, Germany) using 16 µg/mL of pDEST26-CES2 plasmid vector (vector map in Appendice) in a 1:3 ratio of plasmid DNA and PEI. pDEST26-CES2 plasmid vector was acquired from imaGENES (Berlin, Germany) with *hCES2* (geneID 8824) cloned into pDEST26 plasmid (Invitrogen; Carlsbad, U.S.A.).

Cells were harvested 48 h after transfection and chemically lysed with M-PER mammalian extraction reagent (Pierce Biotechnology; Rockford, U.S.A.). The supernatant, obtained by centrifugation at 10,000*g* for 10 min at 4 ^{o}C, was stored at -20 ^{o}C without the addition of protease inhibitors. Total protein concentration was determined using bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology), according to the manufacturer's instructions.

2.2.4 Serum preparation

Human, domestic cat, bovine, domestic dog, and equine sera, from one individual each, were collected without the addition of anticoagulants (Li et al. 2005; Christensen and Stalker 1991). Samples were stored at 4 ^{o}C for 24 h and centrifuged afterward at 3,000 rpm for 15 min. Each sample was aliquoted and stored at -20 ^{o}C until analysis.

2.2.5 Enzymatic assays and inhibition studies

The enzymatic activity reactions were performed in a final volume of 150 µL, under substrate saturation conditions (0.5 mM of 4-MUBA). The addition of each reaction component was performed in ice, and unless otherwise stated, reactions were undertaken at 37 ^{o}C for 8 min, accounting enzyme reaction linearity. Purified and/or recombinant enzymes, cells extracts, and serum were diluted in 50 mM Tris-HCl buffer (pH 7.4).

Stock solutions of the substrates were prepared in ethanol, and stock solutions of the inhibitors were prepared in DMSO. The organic solvent percentage in the final reaction volume never exceeded 5% (*v/v*). A mixture of 90 mM KH_2PO_4 and 40 mM KCl (pH 7.3) was used as the reaction buffer (Pindel et al. 1997).

The inhibition studies were performed by pre-incubation with loperamide (0.25 mM) or BNPP (0.5 mM) at 37 ^{o}C for 15 or 10 min, respectively. The enzymatic reactions were stopped by the addition of an equal volume of ethanol and centrifuged at 10,000*g* for 10 min before analysis.

All samples were injected in triplicate, and average results are presented throughout the text. Intermediate precision was determined by pooled standard deviation, based on replicate independent assays preformed in three different days in two different concentrations and in the presence and absence of inhibitor. Mean *t* tests were performed for evaluation of the statistical significant differences (p = 0.01) between samples, considering intermediate precision.

2.3 Results and Discussion

2.3.1 Capillary electrophoresis method development

There are several methods for the determination of CES activities described in the literature, ranging from spectrophotometric (Hatfield et al. 2008) to HPLC (Ross and Borazjani 2007) and native in-gel hydrolysis assays (Ross and Borazjani 2007); nonetheless, CESs activities appear not to be previously analysed by CE.

There are substrates known to be selective for each CES. As an example, temocapril is hydrolysed by hCES1 but not by hCES2 (Jewell et al. 2007a); in contrast, procaine has been shown to be hydrolysed by hCES2 but not by hCES1 (Takai et al. 1997). Several efforts have been made to identify specific inhibitors that enable separation of the activities of different CESs (Ross et al. 2006; Crow et al. 2010; Wadkins et al. 2005), and there is also an effort to develop new assays that enable the evaluation of inhibitors (Wang et al. 2011). An analytical method was developed for the selective measurement of CES2 activity in any biological sample. The goal was to have a simple, fast, and reliable analytical tool that would enable evaluation of different biological samples. For method development, 4-MUBA was used as a substrate, because of its higher stability than the classical CES substrate *p*-NPA. 4-MUBA is a general CES substrate, and is routinely used in native in-gel hydrolysis assays (Ross and Borazjani 2007; Crow et al. 2007; Ross et al. 2006), and we have found that cholinesterases do not hydrolyse 4-MUBA (data not shown).

To our knowledge, 4-MUBA and its hydrolysis product, 4-MUB, have not been previously analysed using CE, and therefore we proceeded to develop a method optimised for this substrate. When borate-phosphate buffer at pH 8.0 is used, in the CE method, 4-MUB presents an absorbance maximum at 350 nm and has a migration time of about 4.0 min (Figure 2.1). 4-MUBA was not detected. Equipment response was linear for 4-MUB at least in the range of 23.4 µM to 1.5 mM (R^2 = 0.999). Arbitrary absorbance units were converted to concentration using commercially

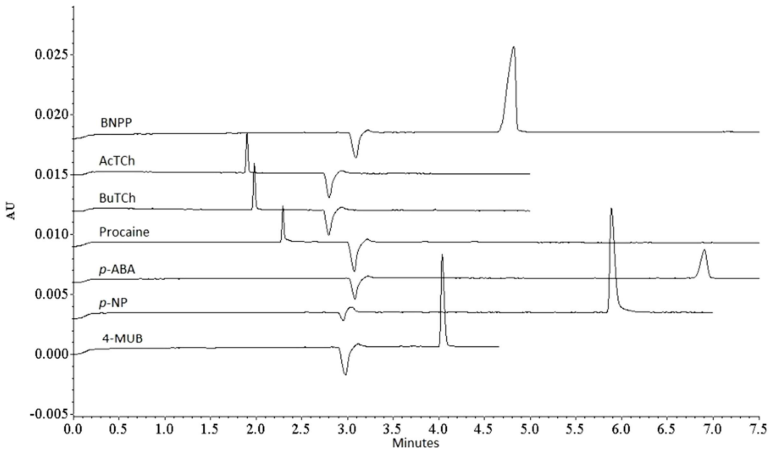
available 4-MUB, as standard. The theoretical limit of detection (13.7 μM) was calculated based on the residual standard deviation of the calibration curve and a signal-to-noise ratio of 3.3. The limit of quantification (23.4 μM) was chosen from the lowest calibration point where the coefficient of variation of repeatability (CV_r) was still under 10% (n=3). CV_r was obtained in the indicated range, 23.4 μM to 1.5 mM 4-MUB, specifically at 1.5 mM and 750, 375, 187.5, 93.8, 46.9, and 23.4 μM. If higher sensitivity is needed, 4-MUB has the advantage of being fluorescent, and thus, using the appropriate detectors, detection and quantification limits can be further improved.

Besides being specific for CESs and since 4-MUBA is not a cholinesterase substrate at the concentrations used, this methodology was found to be compatible with other esterase substrates such as *p*-NPA, procaine, BuTCh and AcTCh, and their corresponding products *p*-NP, *p*-ABA, and thiocholine (TCh), respectively (Figure 2.1). Therefore, the extension of this method to the detection and quantification of the activity of other enzymes may be easily performed. Nonetheless, care should be taken as, for instance, procaine was already shown to be hydrolysed by BuChE (Yuan et al. 2007).

2.3.2 Evaluation of esterase enzymatic activities by CE

Carboxylesterases, AcChEs, and BuChEs are classified as type-B esterases (Aldridge 1953). As previously stated, these enzymes not only show overlapping substrate recognition such as CES2 and BuChE both hydrolysing CPT-11, but they also co-localise in several tissues, such as plasma, intestine, and kidney of several mammals (Liederer and Borchardt 2006). The method was further developed for the ability to discriminate CES2 activity from these other enzymatic activities. Commercial CESs (CES1 and hCES2 recombinant enzyme) and cholinesterases (BuChE and AcChE) were tested toward the hydrolysis of 4-MUBA (Figures 2.2, 2.3a, and data not shown).

A



B

Compound	Wavelength (nm)	Migration time (min)
4-MUBA		N.d.
4-MUB	350	4.0
BNPP	280*	4.8
Loperamide		N.d.
***p*-NPA**		N.d.
***p*-NP**	400	6.0
Procaine	280	2.2
***p*-ABA**	254	6.8
AcTCh	230	2.0
BuTCh	230	2.0
TCh	230	2.5

Figure 2.1 a) Electropherograms of substrates, products, and inhibitors detected by CE. *0.25 mM 4-MUB, 0.5 mM p-NP, 0.1 mM p-ABA, 0.1 mM procaine, 0.5 mM BuTCh, 0.5 mM AcTCh, and 0.25 mM BNPP in a silica capillary 75 µm x 42 cm, 25 ^{o}C, using hydrodynamic injections (0.2 psi for 10 sec). Separation was at +70.0 µA with cathodic migration.* ***b) Maximum absorption wavelengths and migration times.*** *N.d. Not detected. (*) BNPP is also detected at 350 nm.*

For testing 4-MUBA hydrolysis by commercial cholinesterases, 50 ng of each enzyme were incubated in the same conditions as used for CESs. Additionally, cholinesterases were also tested using 0.5 and 1 mM of BuTCh and AcTCh substrates (Figure 2.3b and 2.4). Only CESs were able to hydrolyse the substrate as no significant difference was detected between the spontaneous hydrolysis of 4-MUBA and the hydrolytic activity of the cholinesterases (data not shown).

To distinguish specific hCES2 activity from the activity of the other CESs we propose the use of the hCES2 selective inhibitor loperamide (Jewell et al. 2007b). Although BuChE and AcChE did not hydrolyse 4-MUBA, some biological samples may contain other enzymes that could metabolise this substrate. For this reason, we evaluated BNPP, an irreversible inhibitor specific for CESs (Tanino et al. 2008; Eng et al. 2010).

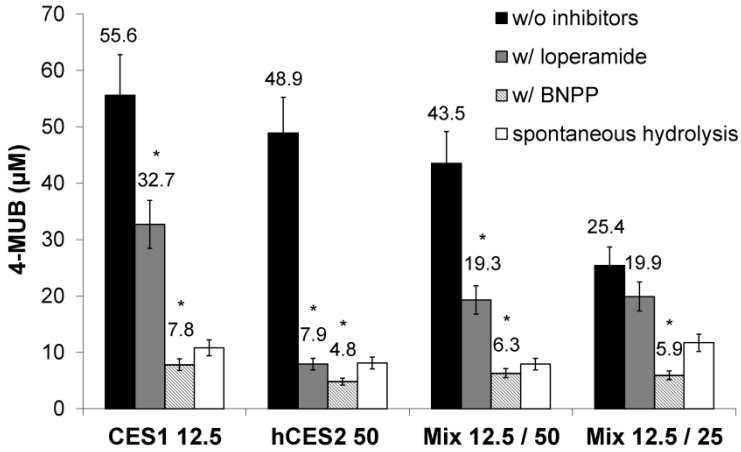


Figure 2.2 Commercial CES activity toward 4-MUBA in the presence and absence of inhibitors. *12.5 ng of CES1 or 50 ng of hCES2 enzymes were incubated with 0.5 mM 4-MUBA in a total reaction volume of 150 µL. Mixtures of the two enzymes included mix 12.5 / 50 (12.5 ng of CES1 and 50 ng of hCES2) and mix 12.5 / 25 ng (12.5 ng of CES1 and 25 ng of hCES2). Pre-incubation with 0.25 mM loperamide or 0.5 mM BNPP was performed when appropriate. The spontaneous hydrolysis of 4-MUBA is also shown. Each result represents the average of three injections, and error bars represent the standard deviation. A * represents a statistical significant difference (p = 0.01) between the enzymatic activities in the presence (grey or dashed bars) and in the absence (black bars) of the inhibitor, based on standard deviation of intermediate precision. The amount of 4-MUB (µM) formed in each sample is mentioned on the top of each bar.*

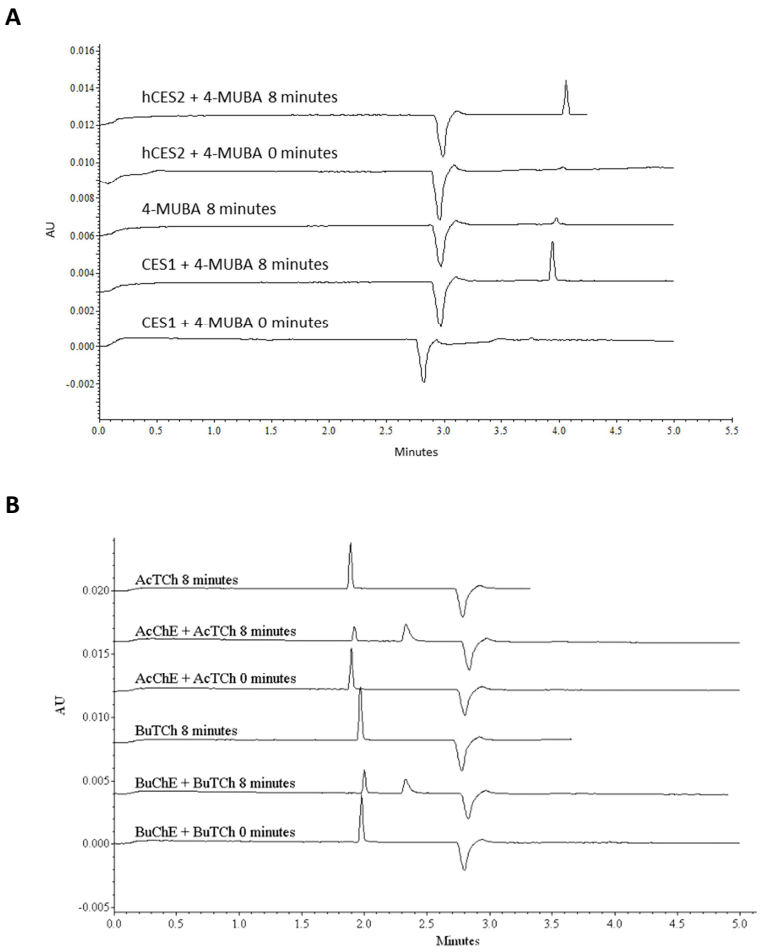


Figure 2.3 Electropherograms of incubation mixtures. a) *12.5 ng of CES1 and 50 ng of hCES2 enzyme reactions were detected by CE at 350 nm. 4-MUBA (0.25 mM) spontaneous hydrolysis was also detected at the same wavelength.* ***b)*** *50 ng of BuChE and 50 ng of AcChE enzyme reactions using BuTCh (0.5 mM) and AcTCh (0.5 mM), respectively, were detected by CE at 230 nm. The substrates (0.5 mM) spontaneous hydrolysis was also detected at the same wavelength.*

Taking advantage of these two cheap and commercially available inhibitors, the relative activity of CES2 can be determined in any biological sample. BNPP also absorbs at 350 nm, and therefore classical spectrophotometric enzymatic assays cannot be used. As different biological samples may contain different amounts of esterases or hCES2, we decided to use high amounts of each inhibitor to ensure that

maximum inhibition is achieved. Lower amounts of inhibitors can be used as demonstrated below (Figure 2.5).

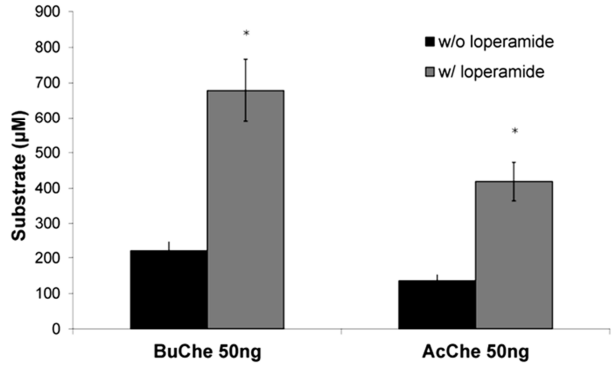


Figure 2.4 BuChE and AcChE activities. *50 ng of each enzyme were incubated with 1 mM BuTCh or AcTCh, respectively, in a total reaction volume of 150 µL. Pre-incubation with 0.25 mM of loperamide was performed when appropriate. Each result represents the average of three injections and the error bars represent the standard deviation. A * represents a statistical significant difference (p = 0.01) between the enzymatic activities in the presence (grey bars) and in the absence (black bars) of the inhibitor, based on standard deviation of intermediate precision.*

In the developed CE method the BNPP absorption spectrum shows a maximum at 280 nm. Despite the fact that it is also detected at 350 nm, the migration time of this inhibitor (4.8 min) does not overlap with that of 4-MUB (4.0 min; Figure 2.1); thus, 4-MUB can be quantified by CE in the presence of BNPP. Using BuChE, AcChE, and their respective substrates BuTCh and AcTCh (Tecles and Cerón 2001), we could observe that loperamide does inhibit both enzymes (Figure 2.4).

Using the described CE method, we could also detect thiocholine (the hydrolysis product of both substrates) at 230 nm as well as both substrates at the same wavelength. Substrates and thiocholine migration times do not overlap, being completely distinguishable (2.0 min for both substrates and 2.5 min for thiocholine, in average; Figure 2.3b).

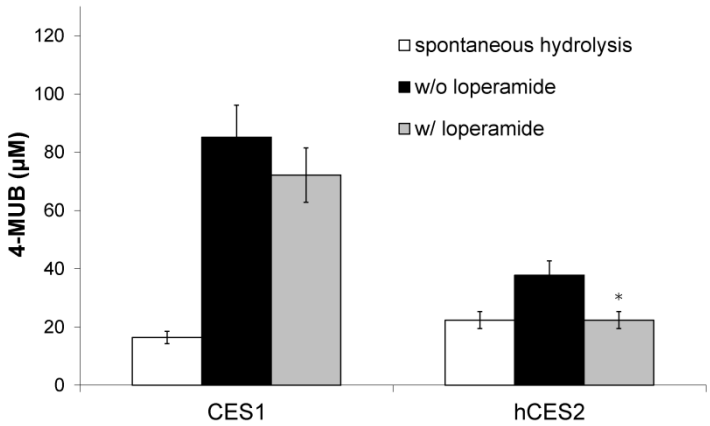


Figure 2.5 Commercial CESs activities towards 4-MUBA in the presence and absence of 50 µM of loperamide. *25 ng of CES1 and 25 ng hCES2 enzymes were incubated with 0.5 mM of 4-MUBA in a total reaction volume of 150 µL. Pre-incubation with 50 µM of loperamide was performed when appropriate. The spontaneous hydrolysis of 4-MUBA is also shown. Each result represents the average of three injections and error bars represent the standard deviation. A * represents a statistical significant difference (p = 0.01) between the enzymatic activities in the presence (grey bars) and in the absence (black bars) of the inhibitor, based on standard deviation of intermediate precision.*

According to available published data (Pindel et al. 1997; Nishi et al. 2006), hCES2 shows a higher catalytic efficiency, defined as $k_{cat}/(k_{dif}*(K_M + [S]))$ (Ceccarelli et al. 2008), toward 4-MUBA than hCES1, but our data is suggesting the opposite. This may simply be due to species differences, to differences in assay conditions, or to differences in enzymes sources. In the described catalytic efficiency formula, besides the enzymatic kinetic parameters, K_M (Michaelis-Menten constant) and k_{cat} (rate constant), k_{dif} (rate for a diffusion-controlled process, approximately 10^9 $M^{-1}.s^{-1}$) and [S] (substrate concentration) are considered. This has been presented as a more correct way of evaluating the performance of different enzymes acting on the same substrate, than the simple k_{cat}/K_M specificity constant (Ceccarelli et al. 2008).

As expected, loperamide completely inhibited the activity of hCES2 but not commercial CES1 (Figure 2.2). On the other hand, the activities of both CESs were completely inhibited by BNPP. We were not expecting to observe CES1 inhibition in the presence of loperamide (Jewell et al. 2007b), but Figure 2.2 disproved this expectation. The IC_{50} and K_i values for these enzymes were previously reported

(Quinney et al. 2005; Williams et al. 2011), showing a dramatic difference between the sensitivity of the enzymes toward loperamide inhibition. In fact, the K_i for hCES2 is practically 1,000 times lower in comparison to the one for hCES1. Thus, this unexpected inhibition verified in CES1 enzyme can be easily overcome by reducing loperamide concentration. We further confirmed this by testing the same concentration of each enzyme with a lower concentration of loperamide, 50 μM. We observed that no significant inhibition is detected in CES1, whereas hCES2 is completely inhibited (Figure 2.5).

In order to demonstrate the ability of the method to distinguish between hCES2 and CES1, we mixed the two enzymes (50 or 25 ng of hCES2 with 12.5 ng of CES1) and performed the same set of assays, ensuring substrate saturation conditions (Figure 2.2). As expected, the incubation of BNPP with both mixtures completely inhibited enzymatic activity. The incubation of each mixture with loperamide caused a decrease in the relative percentage of activity to the same levels, in both mixtures.

Although the protein levels of CESs do not always correlate with their activity levels (Ross and Crow 2007), the proposed methodology enables revealing the relative activities of the enzymes, through the use of the different inhibitors. Other techniques, such as Western blots, estimate the amount of an enzyme in a biological sample but do not provide any information concerning their activity. On the other hand, native in-gel hydrolysis assays permit the detection of enzyme activities, but no direct protein quantification is possible.

This methodology also offers the possibility to determine specific enzyme activities. For this, one has to follow classical enzymatic study rules like ensuring that the assays are performed in the linear range of the enzymatic reaction and the substrate is not at a limiting concentration. In fact, we have quickly determined the linear range of enzymatic reaction for CES1 enzyme (data not shown). In this way, there is no need to appeal to other methodologies usually used, such as

spectrophotometric methods, to determine these parameters. The intermediate precision of the method was determined (CV_i = 12.8%) for two different CES1 concentrations (25 and 12.5 ng) in the presence and in the absence of one of the inhibitors (loperamide) on three different days.

Using the same rationale, the different types of assays that can be performed are unlimited. It is worth noting that the inhibition of BuChE and AcChE by loperamide does not interfere with the detection and/or quantification of hCES2 activity in biological samples as we also saw that BuChE and AcChE do not hydrolyse 4-MUBA.

2.3.3 Carboxylesterase 2 activity determination in complex biological samples

To demonstrate its potential, this methodology was applied to several different biological samples, with different complexities. Human carboxylesterase 2 activity was analysed in extracts from human cells that were transfected with *hCES2* to evaluate the specific activity of the enzyme. Since HEK-293T human cell line only has basal expression of esterases (Xie et al. 2002), the original cells have also been analysed as a control. The linear range of the reaction was assayed and the specific activity of hCES2 was determined both in the overexpressing as well as in the original cells (Figure 2.6).

Total protein in each sample was determined, and all the assays were performed using the same total protein amount. This methodology enabled the use of extremely low amounts of protein, from 3 to 6 µg (Figure 2.6), whereas in classical spectrophotometric assays, the minimum protein amount used in similar assays is 20 µg (Xie et al. 2002), and in native in-gel hydrolysis assays, it reaches 30 µg of total protein amount of tissue extracts (Ross and Crow 2007). We are thus able to use almost 7 times less protein compared to the other methods.

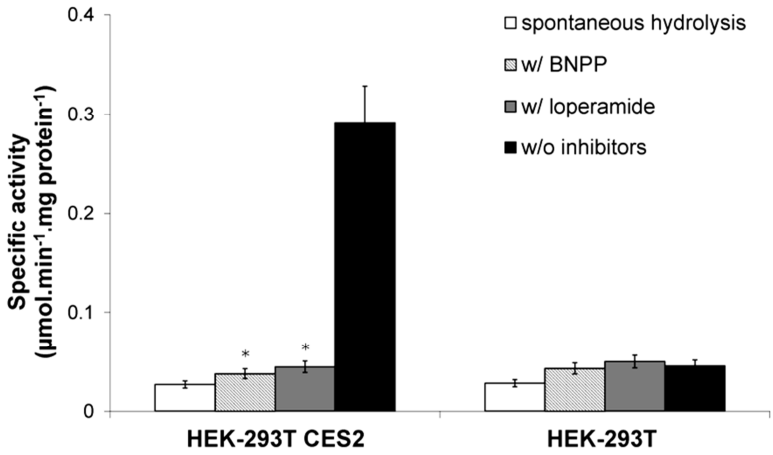


Figure 2.6 Human carboxylesterase 2 specific activity in HEK-293T transfected cells. *HEK-293T cells transfected with hCES2 and non-transfected cells extracts were incubated with 0.5 mM 4-MUBA in a total reaction volume of 150 µL. An amount of 6 µg of total protein was used in each experiment. Pre-incubation with 0.25 mM loperamide or 0.5 mM BNPP was performed when appropriate. The spontaneous hydrolysis of 4-MUBA is also shown. Each result represents the average of three injections, and error bars represent the standard deviation. A * represents a statistical significant difference (p = 0.01) between the enzymatic activities in the presence (grey or dashed bars) and in the absence (black bars) of the inhibitor, based on standard deviation of intermediate precision.*

By evaluating the obtained data, it was possible to conclude the specific activity of hCES2 was increased 15-fold in the transfected cells. In addition, as reported, basal expression of esterases is detected in this cell line. Comparing the specific activities obtained in the presence of loperamide and in the absence of the inhibitor for the transfected cell line it can be concluded that hCES2 activity accounts for 93% of the total specific activity of these transfected cells.

The methodology was further applied in the evaluation of a more complex matrix in terms of enzyme activity. Different mammalian sera, which are known to contain different types and amounts of esterases (Satoh and Hosokawa 1998; Liederer and Borchardt 2006; Tecles and Cerón 2001) were chosen. The serum from one individual of human, bovine, equine, domestic dog, domestic cat, and rat species were thus tested. Using 10% (*v/v*) of serum of each mammal consistent results were obtained for the CESs relative activities (Figure 2.7) to the ones described in the

literature (Li et al. 2005). In fact, we did not observe any CES activity in human or bovine sera and the highest levels of CESs activities were found in rat, equine, and cat sera.

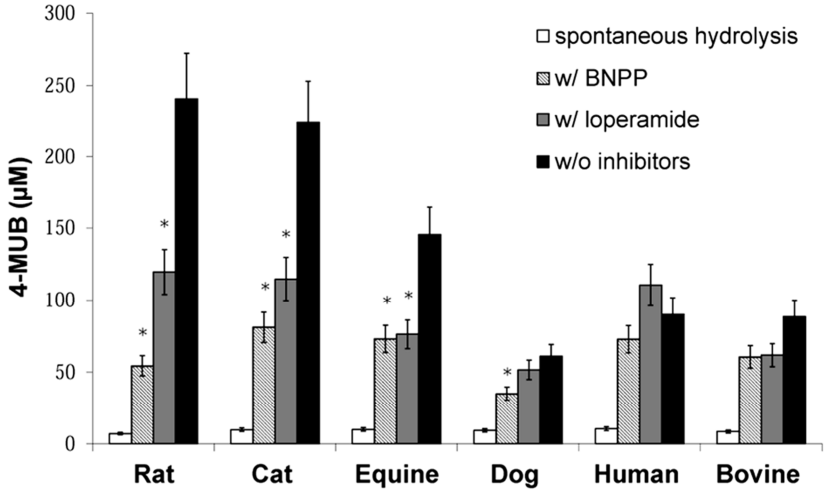


Figure 2.7 Mammalian sera activities toward 4-MUBA in the presence and absence of inhibitors. *An amount of 10% (v/v) of serum from one individual of each mammalian species was incubated with 0.5 mM 4-MUBA in a total reaction volume of 150 µL. Pre-incubation with 0.25 mM loperamide or 0.5 mM BNPP was performed when appropriate. The spontaneous hydrolysis of 4-MUBA is also shown. Each result represents the average of three injections, and error bars represent the standard deviation. A * represents a statistical significant difference (p = 0.01) between the enzymatic activities in the presence (grey or dashed bars) and in the absence (black bars) of the inhibitor, based on standard deviation of intermediate precision.*

In human serum, for example, we can detect esterase activity that is not inhibited by loperamide or BNPP as no significant statistical difference (p = 0.01) is observed between the enzymatic activities obtained in the presence and absence of inhibitors. It is known that human plasma contains four types of esterases, namely, BuChE, AcChE (present in negligible amounts only), paraoxonase, and albumin (Li et al. 2005). We thus hypothesise that the esterase activity detected in human serum is due to one or several of the enzymes that can possibly hydrolyse 4-MUBA but that are not CESs. In fact, albumin is known to hydrolyse this substrate (Crow et al. 2007). Nonetheless, the method proves unaffected by basal activity due to esterases other

than CESs, with inhibition by BNPP and by loperamide being valuable indicators of CESs and CES2 activity, respectively.

Assuming loperamide inhibits CES2-like enzymes from mammalian species as well as it inhibits hCES2, we determined the specific activities of CES2 and total CESs in the different mammals in the linear range of the enzymatic reaction of each serum (Table 2.1). Carboxylesterase 2 activity was detected and quantified in equine, rat, and cat sera. In fact, in equine serum it is apparently the only CES present.

The highest CES2 activity is observed in rat serum; on the contrary, dog serum shows no significant CES2 activity. Once again, our results are consistent with the trends reported in the literature (Li et al. 2005). One should note, however, that our purpose is to demonstrate that we achieve quantifiable and consistent values of specific CES2 activities with our proposed methodology. A deeper evaluation of the CESs present in each mammalian serum would require a higher number of individuals per species.

Table 2.1 Specific activity of CES2 enzymes in mammalian sera [a]

	nmol 4-MUB . min^{-1} . serum % $^{-1}$		
Species	**total serum**	**CES2**	**other CESs**
Equine	0.25 ± 0.01	0.13 ± 0.01	N.D.
Dog	0.10 ± 0.01	N.d.	0.05 ± 0.01
Rat	1.07 ± 0.10	0.82 ± 0.07	0.16 ± 0.03
Cat	0.62 ± 0.07	0.40 ± 0.04	0.06 ± 0.02
Bovine	0.60 ± 0.13	N.d.	N.d.
Human	0.60 ± 0.05	N.d.	N.d.

[a] *Specific activities were determined using 0.5 mM 4-MUBA as described before. Amounts of 10% (v/v) of equine, dog, human, and bovine serum, 1% (v/v) rat serum, and 2.5% (v/v) cat serum were used, to cope with the linear range of the enzymatic reactions. Pre-incubation with 0.25 mM loperamide or 0.5 mM BNPP was performed when appropriate. The spontaneous hydrolysis of 4-MUBA was accounted. Each result represents the average of three injections (± standard deviation). N.d.: not detected.*

To quantify esterase activity and to distinguish CES2 activity from other CESs, we propose the following steps depicted in Figure 2.8. Starting with an unknown sample, an appropriate dilution must be determined in order to detect or to quantify the specific activity of CES2. This can be achieved with 4-MUBA or another substrate hydrolysed by CES2. The ability of an unknown sample to hydrolyse 4-MUBA indicates that it contains esterases able to hydrolyse this substrate; if no activity is detected, the presence of CES2 activity can be excluded and the analysis terminated. Confirmation of activity exclusively due to carboxylesterases is performed in the presence of BNPP, which inhibits these enzymes. If no activity is detected in this step, no CESs are present, and thus analysis will be ended. The analysis of the enzymatic activity in the presence of loperamide enables one to discriminate and quantify the activity of CES2. Loperamide inhibits CES2 but not other CESs, and therefore CESs activities other than CES2 can be determined by the difference between the inhibitions observed using BNPP and loperamide.

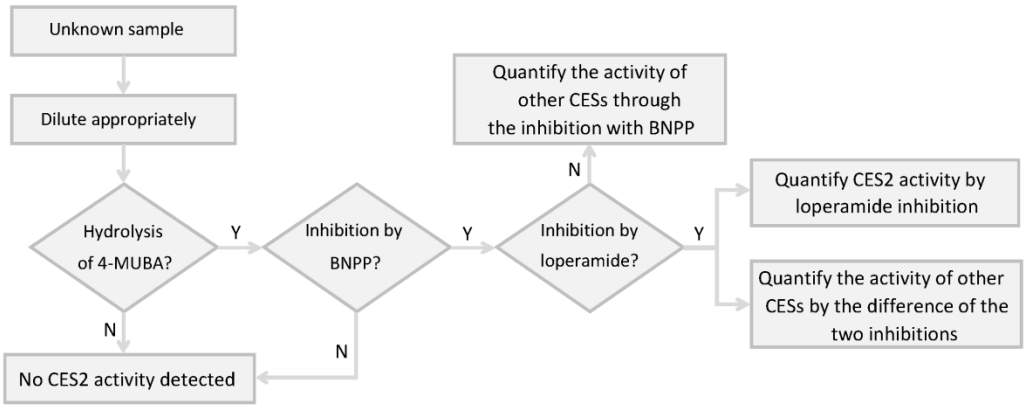


Figure 2.8 Flow diagram for CES2 activity identification and determination.

2.4 Conclusions

Capillary electrophoresis was used for quantification of CESs activities. The methodology proved fast, repeatable, and requires up to 7 times less sample, than classical spectrophotometric methods. Moreover, this approach proved to be applicable even when substrates and products absorb at the same wavelength.

The proposed methodology should be applicable to a wide variety of samples with esterase activity. For example, the analysis and determination of CES1 exclusive activity would only imply the replacement of loperamide by a specific inhibitor of this enzyme such as the partially noncompetitive inhibitor 27- hydroxycholesterol (Tanino et al. 2008). For the analysis of the activities of other esterases, appropriate substrates and inhibitors should be used.

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2.6 References

Adam GC, Vanderwal CD, Sorensen EJ, Cravatt BF (2003) (-)-FR182877 is a potent and selective inhibitor of carboxylesterase-1. *Angew Chem Int Ed* 42: 5480–5484.

Aldridge WN (1953) Serum esterases. I. Two types of esterases (A and B) hydrolyzing *p*-nitrophenyl acetate, propionate and butyrate, and a method for their determination. *Biochem J* 53: 110–117.

Ceccarelli EA, Carrillo N, Roveri OA (2008) Efficiency function for comparing catalytic competence. *Trends Biotechnol* 26: 117-118.

Christensen JM, Stalker DJ (1991) Ibuprofen piconol hydrolysis in vitro in plasma, whole blood, and serum using different anticoagulants. *Pharm Sci* 80: 29–31.

Crow JA, Borazjani A, Potter PM, Ross MK (2007) Hydrolysis of pyrethroids by human and rat tissues: examination of intestinal, liver and serum carboxylesterases. *Toxicol Appl Pharmacol* 221: 1–12.

Crow JA, Herring KL, Xie S, Borazjani A, Potter PM, Ross MK (2010) Inhibition of carboxylesterase activity of THP1 monocytes/macrophages and recombinant human carboxylesterase 1 by oxysterols and fatty acids. *Biochim Biophys Acta* 1801: 31–41.

Elbashir AA, Aboul-Enein HY (2010) Applications of capillary electrophoresis with capacitively coupled contactless conductivity detection (CE-C4D) in pharmaceutical and biological analysis. *Biomed Chromatogr* 24: 1038–1044.

Eng H, Niosi M, McDonald TS, Wolford A, Chen Y, Simila ST, Bauman JN, Warmus J, Kalgutkar AS (2010) Utility of the carboxylesterase inhibitor bis-*para*-nitrophenylphosphate (BNPP) in the plasma unbound fraction determination for a hydrolytically unstable amide derivative and agonist of the TGR5 receptor. *Xenobiotica* 40: 369–380.

Fleury-Souverain S, Vernez L, Weber C, Bonnabry P (2009) Use of capillary electrophoresis coupled to UV detection for a simple and rapid analysis of pharmaceutical formulations in a quality control laboratory in a hospital pharmacy. *Eur J Hosp Pharm* 3: 53–60.

Hatfield JM, Wierdl M, Wadkins RM, Potter PM (2008) Modifications of human carboxylesterase for improved prodrug activation. *Expert Opin Drug Metab Toxicol* 4: 1153-1165.

Hicks LD, Hyatt JL, Stoddard S, Tsurkan L, Edwards CC, Wadkins RM, Potter PM (2009) Improved, selective, human intestinal carboxylesterase inhibitors designed to modulate 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin. (Irinotecan; CPT-11). *J Med Chem* 52: 3742-3752.

Holmes RS, Cox LA, VandeBerg JL (2009) Horse carboxylesterases: evidence of six *CES1* and four families of *CES* genes on chromosome 3. *Comp Biochem Physiol Part D Genomics Proteomics* 4: 54-65.

Holmes RS, Wright MW, Laulederkind SJ, Cox LA, Hosokawa M, Imai T, Ishibashi S, Lehner R, Miyazaki M, Perkins EJ, Potter PM, Redinbo MR, Robert J, Satoh T, Yamashita T, Yan B, Yokoi T, Zechner R, Maltais LJ (2010) Recommended nomenclature for five mammalian carboxylesterase gene families: human, mouse and rate genes and proteins. *Mamm Genome* 21: 427–441.

Jewell C, Ackermann C, Payne NA, Fate G, Voorman R, Williams FM (2007a) Specificity of procaine and ester hydrolysis by human, minipig, and rat skin and liver. *Drug Metab Dispos* 35: 2015–2022.

Jewell C, Prusakiewicz JJ, Ackermann C, Payne NA, Fate G, Voorman R, Williams FM (2007b) Hydrolysis of a series of parabens by skin microsomes and cytosol from human and minipigs and in whole skin in short-term culture. *Toxicol Appl Pharmacol* 225: 221–228.

Kubo T, Kim SR, Sai K, Saito Y, Nakajima T, Matsumoto K, Saito H, Shirao K, Yamamoto N, Minami H, Ohtsu A, Yoshida T, Saijo N, Ohno Y, Ozawa S, Sawada J (2005) Functional characterization of three naturally occuring single nucleotide polymorphisms in the *CES2* gene encoding carboxylesterase 2 (HCE-2). *Drug Metab Dispos* 33: 1482–1487.

Li B, Sedlacek M, manoharan I, Boopathy R, Duysen EG, Masson P, Lockridge O (2005) Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. *Biochem Pharmacol* 70: 1673-1684.

Liederer BM, Borchardt RT (2006) Enzymes involved in the bioconversion of ester-based prodrugs. *J Pharm Sci* 95: 1177-1195.

Moura J, Simplício AL (2010) Electrophoretically mediated microanalysis for the evaluation of interspecies variation in cholinesterase metabolism. *Electrophoresis* 14: 2374–2376.

Na K, Lee EY, Lee HJ, Kim KY, Lee H, Jeong SK, Jeong AS, Cho SY, Kim SA, Song SY, Kim KS, Cho SW, Kim H, Paik YK (2009) Human plasma carboxylesterase 1, a novel serologic biomarker candidate for hepatocellular carcinoma. *Proteomics* 9: 3989–3999.

Nishi K, Huang H, Kamita SG, Kim IH, Morisseau C, Hammock BD (2006) Characterization of pyrethroid hydrolysis by the human liver carboxylesterases hCE-1 and hCE-2. *Arch Biochem Biophys* 445: 115–123.

Pindel EV, Kedishvili NY, Abraham TL, Brzezinski MR, Zhang J, Dean RA, Bosron WF (1997) Purification and cloning of a broad substrate specificity human liver carboxylesterase that catalyzes the hydrolysis of cocaine and heroin. *J Biol Chem* 272: 14769–14775.

Quinney SK, Sanghani SP, Davis WI, Hurley TD, Sun Z, Murry DJ, Bosron WF (2005) Hydrolysis of capecitabine to 5'-deoxy-5-fluorocytidine by human carboxylesterases and inhibition by loperamide. *Pharmacol Exp Ther* 313: 1011–1016.

Ross MK, Borazjani A (2007) Enzymatic activity of human carboxylesterases. *Curr Protoc Toxicol* 33: 4.24.1–4.24.14.

Ross MK, Borazjani A, Edwards CC, Potter PM (2006) Hydrolytic metabolism of pyrethroids by human and other mammalian carboxylesterases. *Biochem Pharmacol* 71: 657–669.

Ross MK, Crow JA (2007) Human carboxylesterases and their role in xenobiotic and endobiotic metabolism. *J Biochem Mol Toxicol* 21: 187–196.

Satoh T, Hosokawa M (1998) The mammalian carboxylesterases: from molecules to functions. *Annu Rev Pharmacol Toxicol* 38: 257–288.

Satoh T, Hosokawa M (2006) Structure, function and regulation of carboxylesterases. *Chem Biol Interact* 162: 195-211.

Takai S, Matsuda A, Usami Y, Adachi T, Sugiyama T, Katagiri Y, Takematsu M, Hirano K (1997) Hydrolytic profile for ester- or amide-linkage by carboxylesterases pI 5.3 and 4.5 from human liver. *Biol Pharm Bull* 20: 869–873.

Tang ZM, Wang ZY, Kang JW (2007) Screening of acetylcholinesterase inhibitors in natural extracts by CE with electrophoretically mediated microanalysis technique. *Electrophoresis* 28: 360-365.

Tanino T, Nawa A, Miki Y, Iwaki M (2008) Enzymatic stability of 2'-ethylcarbonate-linked paclitaxel in serum and conversion to paclitaxel by rabbit liver carboxylesterase for use in prodrug/enzyme therapy. *Biopharm Drug Dispos* 29; 259–269.

Tecles F, Cerón JJ (2001) Determination of whole blood cholinesterase in different animal species using specific substrates. *Res Vet Sci* 70: 233–238.

Wadkins RM, Hyatt JL, Wei X, Yoon KJ, Wierdl M, Edwards CC, Morton CL, Obenauer JC, Damodaran K, Beroza P, Danks MK, Potter PM(2005) Identification and characterization of novel benzyl (diphenylethane-1,2-dione) analogue as inhibitors of mammalian carboxylesterases. *J Med Chem* 48: 2906–2915.

Wang J, Williams ET, Bourgea J, Wong YN, Patten CJ (2011) Characterization of recombinant human carboxylesterases: fluorescein diacetate as a probe substrate for human carboxylesterase 2. *Drug Metab Dispos* 39: 1329-1333.

Williams ET, Wang H, Wrighton SA, Qian YW, Perkins EJ (2010) Genomic analysis of the carboxylesterases: identification and classification of novel forms. *Mol Phylogenet Evol* 57: 23-34.

Williams ET, Bacon JA, Bender DM, Lowinger JJ, Guo WK, Ehsani ME, Wang X, Wang H, Qian YW, Ruterbories KJ, Wrighton SA, Perkins EJ (2011) Characterization of the expression and activity of carboxylesterases 1 and 2 from the beagle dog, cynomolgus monkey, and human. *Drug Metab Dispos* 39: 2305-2313.

Xie M, Yang D, Liu L, Xue B, Yan B (2002) Human and rodent carboxylesterases: immunorelatedness, overlapping substrate specificity, differential sensitivity to serine enzyme inhibitors, and tumor-related expression. *Drug Metab Dispos* 30: 541-547.

Xu G, Zhang W, Ma MK, McLeod HL (2002) Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of irinotecan. *Clin Cancer Res* 8: 2605-2611.

Yuan J, Yin J, Wang E (2007) Characterization of procaine metabolism as probe for the butyrylcholinesterase enzyme investigation by simultaneous determination of procaine and its metabolite using capillary electrophoresis with electrochemiluminescence detection. *J Chromatogr A* 1154: 368-372.

Zhu W, Song L, Zhang H, Matoney L, LeCluyse E, Yan B (2000) Dexamethasone differentially regulates expression of carboxylesterase genes in humans and rats. *Drug Metab Dispos* 28: 186–191.

SECTION 3

Production and characterisation of human recombinant CES2

Adapted from:

Lamego J, Cunha B, Peixoto C, Sousa MF, Simplício AL, Coroadinha AS (2012) Carboxylesterase 2 production and characterization in human cells: new insights into enzyme oligomerization and activity. *Appl Microbiol Biotechnol*; doi 10.1007/s00253-012-3994-3.

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Abstract

Human carboxylesterase 2 (hCES2), the main carboxylesterase expressed in human intestine, is an increasingly important enzyme in anti-cancer combined therapies for the treatment of different pathologies like colon adenocarcinoma and malignant glioma. The production of human recombinant CES2, in human embryonic kidney cells (HEK-293T cells) using serum-free media, is herein described. Carboxylesterase 2 secretion to the media was achieved by the simple addition of an in-frame C-terminal 10× histidine tag (CES2-10xHis) without the need for additional N-terminal signal sequences or the mutation or deletion of the C-terminal HTEL motif responsible for retaining the protein in the lumen of endoplasmic reticulum. This secretion allowed a four-fold increase in hCES2 production. The characterisation of human recombinant CES2 showed this protein exists in additional active and inactive forms than the described 60 kDa monomer.

Contents

3.1 Introduction

Carboxylesterase 2 (CES2) is one of the most abundant carboxylesterases (CESs) in humans (Crow et al. 2007; Satoh and Hosokawa 2006; Schwer et al. 1997). Mammalian CESs are a subset of esterases (Liederer and Borchardt 2006) able to hydrolyse ester containing drugs and prodrugs as well as pesticides such as pyrethroids (Hicks et al. 2009; Holmes et al. 2009). These enzymes are present in diverse tissues such as the liver, testis, small intestine, kidney, and lung, intra or extracellularly depending on the species (Holmes et al. 2009; Satoh and Hosokawa 2006).

Human CESs are not found in the plasma (Li et al. 2005). They are located intracellularly due to the existence of a C-terminal retention sequence composed of four conserved amino acid residues: HTEL, QEDL, and HIEL for hCES2, carboxylesterase 3 (hCES3) and carboxylesterase 1 (hCES1), respectively. This motif enables the retention of CESs in the lumen of the endoplasmic reticulum (ER; Holmes et al. 2010; Satoh and Hosokawa 2006). In humans, hCES2 is found at highest levels in the liver and intestine (Schwer et al. 1997; Crow et al. 2007). It is described as being a very unstable 60 kDa monomeric enzyme (Ross and Borazjani 2007; Pindel et al. 1997).

Carboxylesterase 2 is receiving increased attention due to its potential role in anti-cancer therapies for the treatment of different pathologies such as colon carcinoma (Oosterhoff et al. 2002) and malignant glioma (Tyminski et al. 2005). Also, it has been recommended to monitor hCES2 expression levels before the treatment of human lung cancer with specific drugs, such as Irinotecan (CPT-11; Satoh and Hosokawa 2006). Carboxylesterase 2 has also been used in combined enzyme–drug therapies, where tumour specific activation (Oosterhoff et al. 2002) enables drug dosage reduction and consequently the decrease of its severe adverse side effects (Uchino et al. 2008).

To date, only one human CES crystallographic structure is available. The human carboxylesterase 1 structure, known since 2003, showed that this enzyme is found as active trimers composed of approximately 60 kDa monomers, but the enzyme was also shown to exist as hexamers. In fact, through crystallographic and atomic force microscopy (AFM) studies, hCES1 was shown to exist in trimer–hexamer equilibrium. This equilibrium can be shifted towards the trimer or the hexamer depending on the substrate present (Bencharit et al. 2003). Moreover, hCES1 was also shown to be active as dimers (Takai et al. 1997). Not as much is known about hCES2, which, before the present work had only been demonstrated to be active in its monomeric form (Vistoli et al. 2010). A better knowledge of hCES2 and other hCESs structural features will be crucial for the understanding of their properties as well as the well-described similarities and differences in their behaviour towards different substrates and inhibitors (Bencharit et al. 2003; Fleming et al. 2005).

The recombinant expression of different CESs, from rodents to humans, has been reported in the literature, using yeast (Morton and Potter 2000; Lange et al. 2001), insect (Morton and Potter 2000; Sun et al. 2004; Schiel et al. 2007;Wadkins et al. 2005), and mammalian cells (Morton and Potter 2000; Uchino et al. 2008; Xie et al. 2002). Moreover, different purification strategies have been applied (Morton and Potter 2000; Sun et al. 2004), with the most rapid and versatile being immobilized metal affinity chromatography (IMAC; Williams et al. 2008; Bornhorst and Falke 2003; Waugh 2005). Recombinant protein production using mammalian cells is becoming increasingly frequent, as they are able to perform post-translational modifications, unlike what occurs in prokaryotes. The usage of transient gene expression systems is a valuable tool for decreasing the manufacture time. The choice of the mammalian cell line is a key feature in this process. Presently, the most used mammalian cell lines for large scale gene expression of recombinant proteins are Chinese hamster ovary (CHO) and human embryonic kidney (HEK-293) cells (Pham et al. 2006). This work describes and characterises the manufacturing of human recombinant CES2 using

suspension adapted HEK-293T cells. Carboxylesterase 2 is shown to be secreted by the addition of an in-frame C-terminally localised 10× histidine tag, without the need of additional N-terminal signal sequences or the modification or deletion of the ER retention sequence. Moreover, the purification of human recombinant CES2 and its further characterisation through biochemical techniques unravelled new fundamental features, the presence of oligomeric active and inactive forms.

3.2 Materials and Methods

3.2.1 Materials

Carboxylesterase 1 enzyme (esterase from porcine liver) was from Sigma (Buchs, Switzerland). Endoglycosydase H (Endo H) enzyme was from Roche (Mannheim, Germany). N-glycosidase F (PNGase F) was from Prozyme (Hayward, U.S.A.). Ribonuclease B (RNase B) was from Sigma (Schnelldorf, Germany).

4-Methylumbelliferyl acetate (4-MUBA) (≥98%), 4- methylumbelliferone (4-MUB) (≥98%), Trizma hydrochloride (>99%), Trizma base (>99.9%), Tris-buffered saline (p.a.) and imidazole (≥99.5%) were from Sigma (St. Louis, U.S.A.). Potassium chloride (≥99.5%), brefeldin A (99.9%) and bis-*p*-nitrophenyl phosphate (BNPP) (>99%) were from Aldrich (Steinheim, Germany). Sodium acetate (≥99.0%) and loperamide hydrochloride (p.a.) were from Fluka (Seelze, Germany). Potassium dihydrogen phosphate (≥99%), disodium tetraborate (≥99.5%) and ethanol (99.5%) were from Panreac (Barcelona, Spain). Sodium chloride (≥99.5%), disodium hydrogen phosphate (≥99.5%), sodium dihydrogen phosphate (>99%), methanol (≥99.9%), albumin fraction V (≥98.0%), nickel sulphate (extra pure), Tween 20 (for synthesis) and skim milk (for microbiology) were from Merck (Darmstadt, Germany). Dimethyl sulphoxide (95%) was from Lab-Scan (Dublin, Ireland). Stock solutions of substrates, products and inhibitors were prepared in ethanol. Milli-Q water (Direct-Q 3 UV

ultrapure water purification system from Millipore; Billerica, U.S.A.) was used for the preparation of all working and buffer solutions.

3.2.2 Cells and media

Human embryonic kidney cells (HEK-293T; ATCC CRL-11268) were maintained in suspension cultures using 125-mL Erlenmeyer flasks (Corning; Amsterdam, The Netherlands), with 20 mL of FreeStyle 293 Expression Medium (serum free, animal origin free, chemically defined) from Gibco (Grand Island, U.S.A.), at 37 °C in a humidified atmosphere containing 8% (*v/v*) CO_2 in air, using orbital agitation (130 rpm). Cells were routinely sub cultured twice a week, using an inoculum of 0.5×10^6 cell.mL^{-1}. Cell concentration and viability were determined with a Fuchs–Rosenthal counting chamber (Marienfeld; Lauda-Königshofen, Germany) using trypan blue (Gibco; Carlsbad, U.S.A.) staining.

3.2.3 Plasmids

For the generation of pCI-neo-CES2 and pCI-neo-CES2-10xHis mammalian expression vectors, human *CES2* gene (geneID 8824) and human recombinant *CES2* gene synthetically synthesized with a C-terminal 10xHis tag (GeneArt; Regensburg, Germany) were cloned, respectively, in pCI-neo plasmid (Promega; Madison, U.S.A.) using *SalI* and *NotI* restriction endonucleases (New England Biolabs; Ipswich, U.S.A.). For the formation of a full transcript containing the C-terminal histidine tag, the stop codon of *CES2* gene was deleted. Both constructs were fully sequenced to verify the integrity of *CES2* gene. Production and purification of both expression vectors were performed and evaluated according to standard molecular biology techniques.

3.2.4 Human recombinant CES2-10xHis protein production

For the establishment of the best transfection conditions, HEK-293T cells were transiently transfected with pCI-neo-CES2 or pCI-neo-CES2-10xHis plasmids in 500 mL

Erlenmeyer flasks, containing 90 mL of FreeStyle 293 Expression Medium. Briefly, cells were seeded at 1×10^6 cell.mL^{-1} and allowed to grow for 3 to 6 h. Chemical transient transfection was performed with polycation polyethylenimine (PEI; Polysciences; Eppelheim, Germany). Different plasmid DNA concentrations were tested: 2, 5, 10 and 20 µg.mL^{-1}. Plasmid DNA and PEI were used in a 1:3 ratio and prepared in 10% of the total volume of used media. Cell growth and viability was followed throughout a time period of 96 h. Ten-millilitre samples were taken at 24, 48, 72, and 96 h post-transfection (hpt). Cells were harvested by centrifugation at 200*g*, for 10 min and lysed with 200 µL of M-PER mammalian extraction reagent (Pierce Biotechnology; Rockford, U.S.A.) and further clarified at 10,000*g* for 10 min at 4 °C. Both cell extracts and supernatant media were stored at −20 °C, without the addition of protease inhibitors. Each transfection experiment was independently repeated at least twice.

For the establishment of the best purification conditions, HEK-293T cells were cultured in 500 mL Erlenmeyer flasks, with 90 mL of FreeStyle 293 Expression Medium and transiently transfected as described above, using 5 µg.mL^{-1} of pCI-neo-CES2-10xHis plasmid. At least three 500 mL Erlenmeyer flasks were used per experiment. Cell growth and viability were followed throughout 96 h. Cells were harvested 96 hpt, by centrifugation at 200*g*, for 10 min. The culture media, containing the soluble human recombinant CES2-10xHis protein, was analysed and stored at −20 °C until the beginning of the purification process.

For larger scale production, a 5 L working volume bioreactor (BIOSTAT DCU-3, Sartorius Stedim Biotech; Aubagne, France) was inoculated at 0.5×10^6 cell.mL^{-1} of HEK-293T cells. Transient transfection was performed as described above, using 5 µg.mL^{-1} of pCI-neo-CES2-10xHis plasmid. The bioreactor was operated in batch mode, equipped with two 6-D Rushton impellers. The pO_2 was set at 40% of air saturation and sequentially controlled varying the agitation rate (60 to 210 rpm) and the oxygen partial pressure in the gas inlet (0–100%). pH was controlled at 7.2 using CO_2 and 1 M

sodium bicarbonate addition. Culture gassing was performed using a ring sparger at a constant gassing rate of 0.01 vvm. The temperature was kept at 37 °C by water recirculation in the vessel jacket. Data acquisition and process control were performed using MFCS/Win (Multi Fermenter Control Supervisory for Windows) control and data acquisition software (SCADA, Sartorius Stedim Biotech).

3.2.5 Impairment of the protein secretion pathway with brefeldin A

Inhibition of the protein secretion pathway was performed with brefeldin A antibiotic, as described before (Potter et al. 1998), with some modifications. HEK-293T cells were transiently transfected with 5 $\mu g.mL^{-1}$ of pCI-neo-CES2-10xHis or pCI-neo-CES2 expression vectors, as described above. At 24, 48, 72 and 96 hpt, two samples (of 10 mL) from each transfection were centrifuged at 200*g* for 10 min. Cells were resuspended in FreeStyle 293 Expression medium with or without 10 $\mu g.mL^{-1}$ of brefeldin A. Cells were incubated according to the same conditions described above for 4 h. Immediately after the incubation period, cell extracts and supernatant were processed as described above and CES activity was evaluated by spectrophotometry.

3.2.6 Purification of human recombinant CES2-10xHis protein

Purification of human recombinant CES2 was performed by affinity chromatography in an ÄKTA explorer 10S system (GE Healthcare; Little Chalfont, UK), using a 5-mL HiTrap Chelating High Performance column (GE Healthcare), loaded with 100 mM of nickel sulphate and equilibrated with 20 mM sodium phosphate buffer, pH 7.4, containing 20 mM imidazole and 500 mM sodium chloride.

Two hundred millilitres of cell supernatant, obtained as described above and further clarified by centrifugation at 10,000*g*, for 10 min at 4 °C, were diluted in an equal volume of 20 mM sodium phosphate buffer, pH 7.4, containing 20 mM imidazole and 500 mM sodium chloride, and loaded onto the column. A washing step was performed with the same buffer until stabilisation of the baseline at 280 nm.

Elution of the His-tagged bound proteins was performed with a two-step imidazole gradient, from 10 to 500 mM. The first lower step was performed from 10 until 250 mM of imidazole in ten column volumes. The following step, until 500 mM of imidazole, was performed with five column volumes. A flow rate of 5 $mL.min^{-1}$ was used.

Buffer exchange and protein concentration were performed with 20 mM sodium acetate buffer (pH 5, containing 600 mM sodium chloride) to all eluted fractions using Vivaflow cassettes (Sartorius Stedim Biotech; Goettingen, Germany) with a membrane cut-off of 30 kDa. After imidazole removal, all samples were sterilized by filtration, using Acrodisc 0.2-µm low protein binding syringe filter (Pall Life Sciences; Ann Arbor, U.S.A.). After the addition of 20% (*v/v*) glycerol, all samples were aliquoted and stored at −80 °C. All fractions were analysed by SDS-PAGE and Western blot as described below.

3.2.7 Glycosylation analysis

Purified CES2-10xHis (100 ng) was incubated with 2.5 mU of Endo H or 1 mU of PNGase F overnight (ON) at 37 °C,, according to the manufacturer's instructions. Negative controls, without deglycosylation enzymes, were performed. RNAse B was used as positive control of both enzymatic deglycosylations. The reaction products were precipitated with four volumes of ice-cold ethanol and analysed by Western blot as detailed below.

3.2.8 SDS- and native PAGE

Total protein concentration was determined using bicinchoninic acid protein assay (Pierce Biotechnology), according to the manufacturer's instructions. For the cell culture supernatant, total protein concentration was not determined due to the interference of the medium components in the protein quantification methods. When

needed, samples were concentrated using Microcons (Millipore) with a 10 kDa cut-off.

Unless otherwise stated, for SDS-PAGE electrophoresis, the same total protein amount (50 µg) was loaded onto NuPAGE 4–12% (*w/v*) bis-tris acrylamide gels (Invitrogen). All samples were denatured and reduced for 10 min at 70 °C, according to the manufacturer's instructions. Electrophoresis was performed in XCell SureLock mini-cell system (Invitrogen) using MOPS SDS running buffer (Invitrogen), according to the manufacturer's instructions. Proteins were stained with Instant Blue (Expedeon; Harston, U.K.) or with SilverXpress staining kit (Invitrogen), according to each manufacturer's instructions.

Electrophoresis under native conditions was performed using native 4–16% (*w/v*) bis-tris acrylamide gels (Invitrogen), according to the manufacturer's instructions, at 4 °C. In-gel activity assay with the substrate 4-MUBA was performed as described before (Ross et al. 2006; Ross and Borazjani 2007) with some modifications. In brief, the gel was washed in Milli-Q water for 10 min and incubated with 0.01% (*w/V*) of 4-MUBA in sodium phosphate buffer (pH 7.0) for 20 min with gentle agitation. The gel was visualized under UV transillumination, and the resulting fluorescence was acquired with ChemiDoc (Bio-Rad; Hercules, U.S.A.) and quantified with ImageJ open source software (NIH; Bethesda, U.S.A.).

3.2.9 Western blot analysis

Western blots were performed using denaturing, non-denaturing and native conditions. For denaturing conditions, SDS-PAGE electrophoresis was performed as described above. For non-denaturing conditions, SDS-PAGE electrophoresis was performed as described above with the exception that the samples were not reduced or denatured. For native conditions, native PAGE electrophoresis was performed as described above. The gels were transferred to nitrocellulose (GE Healthcare) or to

PVDF (Millipore) membranes and blocked ON with 5% (*w/v*) milk in 0.05% (*v/v*) Tween, Tris-buffered saline solution. Semi-dry transfer was performed using Amersham Hoefer TE 70 transfer unit (GE Healthcare) according to the manufacturer's instructions.

The following primary antibodies were used: rabbit anti-CES2 (1:200), from Sigma and goat anti-CES2 (1:200), from R&D systems (Minneapolis, U.S.A.). Secondary antibodies used were: alkaline phosphatase (AP)-goat anti-rabbit (1:2,000), from Sigma; horseradish peroxidase (HRP)-rabbit anti-goat (1:5,000), from Invitrogen and HRP-donkey anti-rabbit (1:20,000), from GE Healthcare. Detection was performed with NBT/BCIP (Pierce Biotechnology), for AP, or Amersham ECL™ Plus (GE Healthcare), for HRP. Image acquisition was performed with ChemiDoc and quantified using ImageJ. At least three images, acquired with different exposure times, without saturation of the obtained signal were used for quantification. Each experiment was repeated at least twice to confirm the verified pattern.

3.2.10 Spectrophotometric assay for esterase activity

The enzymatic activity assays were performed using the substrate 4-MUBA in 96-well plates. All the components were added on ice to a final reaction volume of 250 µL per well. Reactions were undertaken at 37 °C under substrate saturation conditions. Unless otherwise stated, for cell extract samples, the same total protein amount (10 µg) was used in each assay; for all culture supernatant samples, the same volume (50 µL) was used in each assay. Each sample was diluted in 50 mM Tris–HCl buffer (pH 7.4). A mixture of 90 mM potassium dihydrogen phosphate and 40 mM potassium chloride (pH 7.3) was used as reaction buffer. Stock solutions of the substrate were prepared in ethanol. The organic solvent percentage in the final reaction volume never exceeded 5% (*v/v*).

Measurement of the production of 4-MUB, the hydrolytic product of 4-MUBA, was performed at 350 nm (Pindel et al. 1997) on a SpectraMax 340 plate reader (Molecular Devices; Sunnyvale, U.S.A.). An extinction coefficient of 12.2 $mM^{-1}.cm^{-1}$ (Pindel et al. 1997) was considered. Non-enzymatic hydrolysis was subtracted for each enzymatic reaction. Results of esterase activity are presented as micromoles per minute per millilitre of product formed for cell extracts and culture media, or as CES specific activity (micromoles per minute per milligram of total protein) for the purified fractions. Enzyme kinetic parameters were determined for purified recombinant CES2 through nonlinear regression using GraphPad Prism (GraphPad Software; La Jolla, U.S.A.). Results represent the average and standard deviation of three independent assays, which were performed in triplicate.

3.2.11 Capillary electrophoresis assay for CES activity

For hCES2 specific activity detection and quantification, a capillary electrophoresis (CE) method developed by our group was used (Lamego et al. 2011). In brief, for inhibition studies, each sample was pre-incubated with loperamide (25 µM) or BNPP (500 µM), for 15 and 10 min, respectively, to selectively inhibit total hCES2 or total hCESs activity. The enzymatic reactions were performed as described above. Unless otherwise stated, reactions were stopped after 8 min by the addition of equal quantity (*v/v*) of ethanol and centrifuged at 10,000*g* for 10 min before analysis.

The enzymatic linear range was assessed using different concentrations of each sample. CE separation and detection were performed using a Beckman Coulter (Palo Alto, U.S.A.) P/ACE MDQ capillary electrophoresis system equipped with a diode array detector. 4-MUB, was detected at 350 nm. Non-enzymatic hydrolysis was subtracted for each sample. Arbitrary absorbance units were converted to concentration using commercially available 4-MUB, as standard. Results are presented as the average and standard deviation of three independent assays. Mean *t*

tests were performed for the evaluation of the statistical significant differences (p = 0.05) between samples.

3.3 Results

3.3.1 Evaluation of human recombinant CES2 expression in HEK-293T cells

HEK-293T cells were transiently transfected in suspension cultures with 5 $\mu g.mL^{-1}$ of pCI-neo-CES2-10xHis and pCIneo-CES2 expression vectors in serum-free media and allowed to grow for 24 h before harvesting. Both cell culture supernatant and cells were collected and processed as described in Materials and Methods. The expression profiles of esterases both in cell extracts as well as in the supernatant were evaluated. Esterase activity was detected in HEK-293T cell supernatants when using pCI-neo-CES2-10xHis plasmid but not pCI-neo-CES2 plasmid (Figure 3.1a) as soon as 24 hpt. Considering the total activity of each sample, both intra- and extracellularly, it became clear that more than 80% of the esterase activity was detected in the supernatant of HEK-293T cells transiently transfected with pCI-neo-CES2-10xHis expression vector. Moreover, total expression was higher when pCI-neo-CES2-10xHis transfection is performed. The presence of the enzyme in the supernatant is not due to cell lysis since no significant decrease in cell viability was detected (initial viability 95±4%; viability 24 hpt 90±9%).

The activity of human recombinant CES2 in the supernatant of HEK-293T cells transfected with each expression vector was determined through capillary electrophoresis in the presence of loperamide (a selective inhibitor of hCES2; Williams et al. 2011) to confirm the observed increase in esterase activity was due to hCES2 (Figure 3.1b; Lamego et al. 2011).

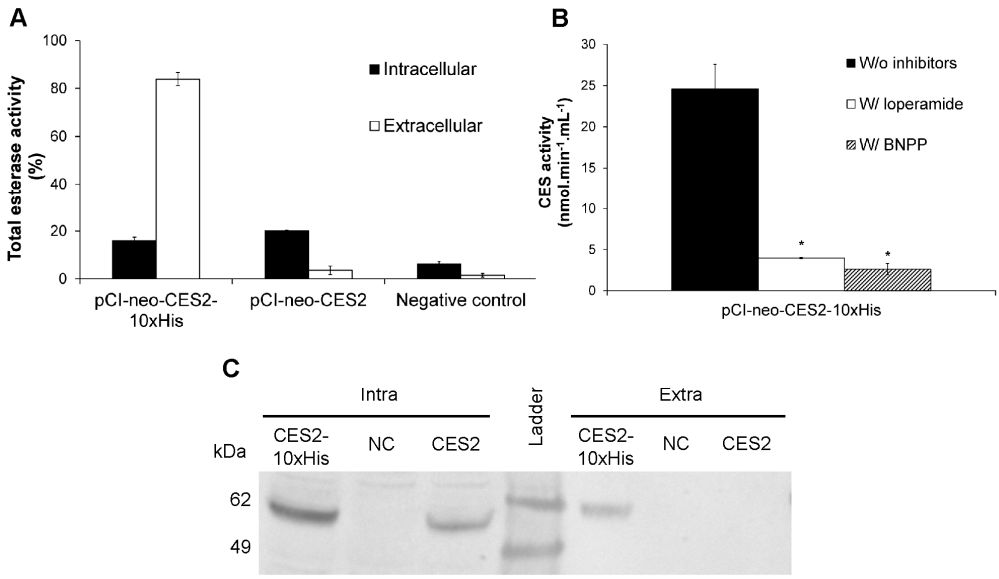

Figure 3.1 Carboxylesterase 2 expression and activity in HEK-293T transiently transfected cells 24 hpt. ***a)*** *Esterase activity towards 4-MUBA evaluated by spectrophotometry both in cell extracts (intracellular) and in cell culture supernatant (extracellular). Activity is shown as the relative percentage to the total esterase activity (intra plus extracellular) of HEK-293T pCI-neo-CES2-10xHis sample, the maximum activity observed. Error bars represent the standard deviation of three independent assays.* ***b)*** *Evaluation of the activity specifically due to hCES2 towards 4-MUBA, in 50 µL cell culture supernatant evaluated by capillary electrophoresis. Each result represents the average of three independent assays, and error bars represent the standard deviation. A * represents a statistical significant difference (p = 0.05) between the enzymatic activities in the absence (black bar) and in the presence of the inhibitors loperamide or BNPP (white or dashed bars, respectively).* ***c)*** *Western blot of pCI-neo-CES2-10xHis (CES2-10xHis) or pCI-neo-CES2 (CES2) transfected and non-transfected (NC, negative control) HEK-293T cell extracts (Intra) and in the respective cell culture supernatant (Extra). Fifty micrograms (total protein) of intracellular and 10 µL of extracellular samples were analysed, using rabbit anti-CES2 and AP-conjugated anti-rabbit antibodies. The 62 (upper) and 49 (lower) kDa bands of the ladder are shown.*

The detection and quantification of the activity of other CESs in the sample were performed by subtracting the activity obtained in the presence of loperamide to the one obtained in the presence of BNPP (an inhibitor of all CESs). If esterases, other than CESs, are present in the sample, their activities will be revealed by the maintenance of activity in the presence of BNPP. The results clearly show that, in the supernatant of HEK-293T cells transiently transfected with pCI-neo-CES2-10xHis, the detected esterase activity, 24 hpt, is mainly due to hCES2. In fact, upon the addition

of loperamide or BNPP, there is no statistically significant difference (p = 0.05) between the obtained activities, meaning that there are no active CESs in the cell culture supernatant other than hCES2. However, the possible existence of other proteins with esterase activity in the cell culture supernatant cannot be ruled out, as a small activity percentage is detected in the presence of BNPP (Figure 3.1b). Carboxylesterase 2 activity accounts for more than 89% of the total enzymatic activity detected in the sample.

The expression of human recombinant CES2 was also confirmed by Western blot. As expected, and in accordance with the obtained results for enzyme activities, hCES2 in HEK-293T cell extracts transfected with both expression vectors was unequivocally detected while no hCES2 was detected in non-transfected control cells (Figure 3.1c). Moreover, human recombinant CES2 was detected in the supernatant of HEK-293T cells when transfected with pCI-neo-CES2-10xHis but not when transfected with pCI-neo-CES2 plasmid, or in the non-transfected control cells. Thus it is showed that by masking the ER retention motif in the C-terminus region (through the addition of a Histidine tag), hCES2 enzyme is secreted without the need to mutate or remove the KDEL retaining motif or add any extra signal peptides at the N-terminal region.

3.3.2 Optimisation of soluble human recombinant CES2 expression

The expression profile of CES2-10xHis in the supernatant of HEK-293T transiently transfected cells was further monitored using different concentrations of plasmid DNA (2, 5, 10, and 20 $\mu g.mL^{-1}$ of pCI-neo-CES2-10xHis expression vector). The evaluation of total enzymatic activity in extracellular culture media throughout time (Figure 3.2a) reveals that 2 $\mu g.mL^{-1}$ of the vector was an insufficient concentration as it gave rise to the lowest detected activities, nearly undetected. On the other hand, 20 $\mu g.mL^{-1}$ of plasmid showed to be too high of a DNA concentration since lower enzymatic activities than at 5 and 10 $\mu g.mL^{-1}$ were detected.

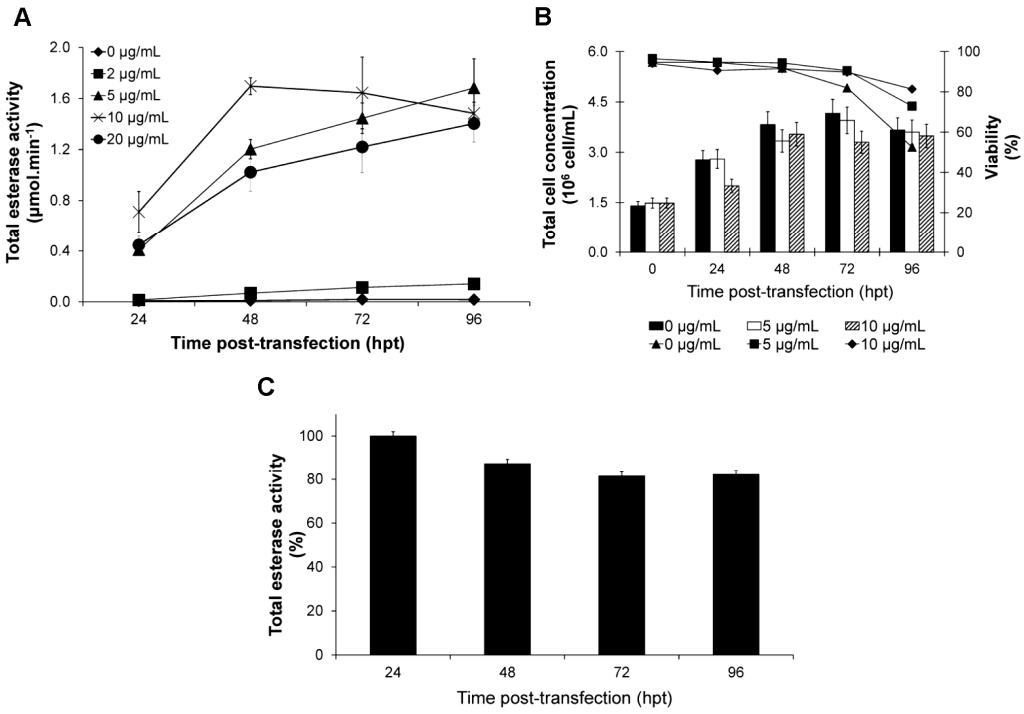

Figure 3.2 Optimisation and evaluation of hCES2 expression in HEK-293T cells transiently transfected with different pCI-neo-CES2-10xHis DNA concentrations. a) *Esterase activity towards 4-MUBA evaluated by spectrophotometry, 24, 48, 72, and 96 hpt with different vector concentrations: 0, 2, 5, 10 and 20 µg.mL^{-1}. Esterase activity is expressed as product formed per time unit (micromoles per minute) in 10 mL of cell culture supernatant. Each result represents the average of three independent assays, and error bars represent the standard deviation.* ***b)*** *Total cell concentration (cell.mL^{-1}) and viability (%) of HEK-293T non-transfected and transfected (5 and 10 µg.mL^{-1}) cells throughout time. Error bars represent a 10% error in cell counting.* ***c)*** *Stability of esterase activity in cell culture medium evaluated by spectrophotometry. Twenty-four hpt supernatant was centrifuged at 200g to remove the cells and incubated for 48, 72, and 96 h at 37 °C and 130 rpm. Decay in esterase activity is shown as the relative percentage to the maximum activity observed at 24 hpt.*

The highest activities were achieved in all assays at 96 hpt with the exception of the 10 µg.mL^{-1} assay where it was achieved at 48 hpt. Thus, transfection with 5 and 10 µg.mL^{-1} of plasmid originated the highest activities although at different culture times. Total cell concentration and viability were also evaluated for these two best DNA conditions (Figure 3.2b). There was no decrease in cell concentration when comparing transfected vs. non-transfected cells. At 96 hpt, both in transfected and non-transfected cells, there was a decrease in cell viability (Figure 3.2b). This suggests

that, at this stage, cell lysis may contribute to the enzymatic activity detected in the cell supernatant. Since other hCESs than hCES2 are expressed intracellularly, these may also contribute to the overall enzyme activity detected in the extracellular culture media.

To test this hypothesis, hCES2 activity was investigated in the culture media of HEK-293T cells transfected with 5 $\mu g.mL^{-1}$ of pCI-neo-CES2-10xHis, by capillary electrophoresis, as detailed above. Carboxylesterase activity in the absence of inhibitors (374.8 ± 23.2 $\mu mol.min^{-1}.mg^{-1}$) shows a dramatic decrease in the presence of loperamide (5.6 ± 2.4 $\mu mol.min^{-1}.mg^{-1}$) and in the presence of BNPP (1.5 ± 0.2 $\mu mol\ min^{-1}.mg^{-1}$). The results show that, as verified for 24 hpt, the detected hCES activity was mainly due to hCES2. In fact, upon the addition of loperamide or BNPP, there was no statistical significant difference (p=0.05) between the obtained activities, meaning that there are no active hCESs other than hCES2 in the supernatant.

To evaluate the stability of the activity of the enzyme in the cell culture supernatant, HEK-293T cells were transiently transfected with 5 $\mu g.mL^{-1}$ of pCI-neo-CES2-10xHis. Cells were removed 24 hpt. The decrease in the enzyme activity in the cell culture supernatant was followed spectrophotometrically until 96 hpt. The results showed a 20% decrease in hCES activity. The activity decrease was more accentuated during the first 24 h after cell removal (Figure 3.2c). Taking these results together, the 5 $\mu g.mL^{-1}$ pCI-neo-CES2-10xHis at 96 hpt was the most appropriate plasmid DNA concentration and harvesting time to obtain the highest amount of active human recombinant CES2-10xHis in the culture media of HEK-293T transiently transfected cells without compromising the enzyme activity and quality. 10 $\mu g.mL^{-1}$ DNA at 48 or 72 hpt would also be suitable, but, since it implied the use of twice the amount of expression vector, the 5 $\mu g.mL^{-1}$ was preferred.

3.3.3 Human recombinant CES2 secretion in HEK-293T cells

Human recombinant CES2-10xHis produced by transient transfection in HEK-293T cells was secreted to the culture media, as shown in the previous sections. This secretion to the supernatant was due to the presence of the C-terminal 10xHistidine tag as no enzyme activity or expression was detected in the culture media of pCI-neo-CES2 transiently transfected HEK-293T cells (Figures 3.1a, c).

It has been previously reported that the expression of mutated human alveolar macrophage CES and rabbit liver CESs, missing the HIEL C-terminal motif, in COS-7 monkey fibroblasts, originates the secretion of these enzymes through the classical ER/Golgi-dependent exocytosis (Potter et al. 1998). To evaluate whether human recombinant CES2-10xHis produced by transient transfection of HEK-293T cells was secreted from the cells via the same pathway, brefeldin A (an antibiotic that inhibits protein secretion through ER/Golgi pathway; Nickel 2010) was used.

The incubation of pCI-neo-CES2-10xHis transiently transfected HEK-293Tcells with brefeldin A inhibited the secretion of the protein as the hCES activity detected in the cell culture media significantly dropped (Figure 3.3). This decrease in extracellular hCES activity was accompanied by an increase in the detected intracellular hCES activity in the brefeldin A treated vs. non-treated cells (data not shown). The basal levels of hCES activity detected in the culture media upon the treatment with brefeldin A correspond to the activity of other esterases towards 4-MUBA, as verified in the previous sections. The incubation of pCI-neo-CES2 transiently transfected HEK-293T cells with the antibiotic was performed as control. As expected, the incubation of these cells with brefeldin A did not produce any alteration in the intracellular or extracellular activity of hCES (data not shown).

The incubation of the two types of transiently transfected HEK-293Tcells with brefeldin A did not originate any decrease in cellular viability in comparison to the non-incubated cells (data not shown).

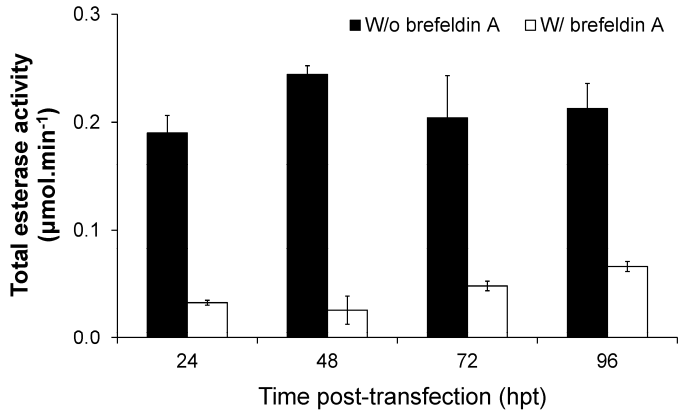

Figure 3.3 Effect of brefeldin A on extracellular esterase activity of pCI-neo-CES2-10xHis transiently transfected HEK-293T cells. *Esterase activity towards 4-MUBA evaluated by spectrophotometry, 24, 48, 72, and 96 hpt with 5 µg.mL^{-1} of the vector with or without a 4-h treatment with 10 µg.mL^{-1} of brefeldin A. Esterase activity is expressed as product formed per time unit (in micromoles per minute) in 10 mL of supernatant culture media. Each result represents the average of three independent assays, and error bars represent the standard deviation.*

These results clearly demonstrate that CES2-10xHis is secreted to the cell milieu through the classical secretory pathway. It can thus be assumed that CES2-10xHis is undergoing its normal processing pathway, entering the ER where it can be properly folded and post-translationally modified.

3.3.4 Characterisation of purified human recombinant CES2-10xHis

Human recombinant CES2-10xHis enzyme was purified by nickel affinity chromatography, as described in Materials and Methods. Two hundred millilitres of the supernatant harvested at 96 h after HEK-293T cells transfection with 5 µg.mL^{-1} of pCI-neo-CES2-10xHis were used to purify the recombinant protein.

A two-step imidazole gradient was used to allow a proper separation of CES2-10xHis from the remaining proteins present in the media. Two fractions were recovered from the purification process at 65 (fraction 1) and 250 mM (fraction 2) of imidazole. Both fractions as well as the flow-through containing the proteins that did not bind to the affinity column were processed immediately for buffer exchange in order to remove imidazole from the samples. All samples were stored at −80 °C with 20% glycerol in

order to preserve protein stability. Protein quantification revealed that eluted fraction 1 had higher protein content (26.4 $\mu g.mL^{-1}$) than fraction 2 (9.3 $\mu g.mL^{-1}$). No significant hCES activity was detected in the flow-through of the column (data not shown), but both eluted fractions showed hCES activity. However, the specific enzymatic activity in fraction 2 (22.8 ± 2.1 $\mu mol.min^{-1}.mg^{-1}$) was five times higher than in fraction 1 (4.3 ± 0.3 $\mu mol.min^{-1}.mg^{-1}$). These fractions were further analysed by SDS-PAGE to evaluate protein purity (Figure 3.4a). Fraction 2 shows a clear and defined band corresponding to the previously described approximately 60 kDa hCES2 weight (Pindel et al. 1997). Moreover, no major contaminants were identified with only two extra faint bands visible in the lane. On the contrary, fraction 1 contains several additional bands with different weights, besides a faint band of approximately 60 kDa, which justifies the higher total protein content and the lower hCES activity detected in comparison to fraction 2.

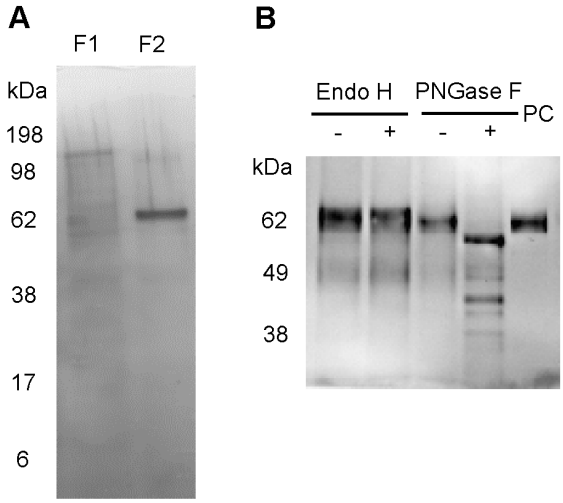

Figure 3.4 Characterisation of human recombinant CES2-10xHis purity after purification. a) *Silver stained SDS-PAGE electrophoresis, for protein purity evaluation. Twenty-one nanograms of total protein of fractions 1 and 2 eluted during the affinity chromatography purification process were used.* ***b)*** *Western blot of purified CES2-10xHis after digestion with Endo H and PNGase F. CES2-10xHis incubated with Endo H or PNGase F buffer (−). Digested CES2-10xHis (+). Positive control—non-digested CES2-10xHis (PC). Goat anti-CES2 and HRP-conjugated antibodies were used.*

To further confirm that the approximately 60 kDa band in fraction 2 corresponds to hCES2, a Western blot using a commercially available anti-CES2 antibody was performed (data not shown). The results indicated that human recombinant CES2 was present in eluted fraction 2 with a higher purity level. To further confirm that no other hCESs were present in this eluted fraction, hCES2 activity was evaluated by capillary electrophoresis, showing once again that no other hCESs were present in this sample (data not shown). Using 4-MUBA as a substrate, K_M and k_{cat} kinetic parameters, of human recombinant CES2 were determined to be $2.5\times10^{-1} \pm 2.8\times10^{-2}$ mM and $1.1\times10^{3} \pm 1.7\times10^{2}$ min^{-1}, respectively. These results, especially the K_M, are in agreement with the ones reported by Pindel et al. (1997) where hCES2 purified from human liver was characterised. As human recombinant CES2 activity was assayed in the same way as described by Pindel et al. (1997), these data suggest that CES2-10xHis shows similar substrate binding characteristics in comparison to native hCES2 and an apparently lower catalytic efficiency that may be due to differences in assay conditions or to differences between the two enzymes

The glycosylation status of the purified protein was also assessed (Figure 3.4b) through the digestion with Endo H, a deglycosylation enzyme that hydrolyses N-glycan chains of the high-mannose type (Tarentino et al. 1978), and PNGase F, a deglycosylation enzyme that hydrolyses N-glycan chains of the complex type (Tarentino and Plummer 1994, Fig. 4b). Purified CES2-10xHis was shown to be post-translationally modified as it is sensitive to PNGase F digestion.

Having CES2-10xHis protein and a commercially available anti-CES2 antibody allowed to estimate CES2-10xHis production as well as the purification process yields. In this way, we estimated the volumetric yield of HEK-293T cells transiently transfected to be 50 $mg.L^{-1}$ of CES2 human recombinant protein. Considering only the CES2-10xHis protein present at a highly pure degree in the eluted fraction 2, we estimate a 2% yield in this purification process. The obtained purification yield is in

agreement with recently reported yields for the purification of recombinant CES2 produced in insect cells, although strategies other than IMAC were applied (Hatfield et al. 2010). The possible reasons for the low yields of hCES2 throughout the different purification strategies will be further discussed in the following sections. CES2-10xHis production was scaled up to 5 L using a bioreactor. All the conditions optimised in Erlenmeyer flasks were kept. Cell viability and hCES activity were followed kinetically. Cell concentration and viability profiles in the bioreactor were comparable to the ones obtained in Erlenmeyer flasks. Taken together, these results show that CES2-10xHis production obtained through transient transfection of suspension adapted HEK-293T cells is scalable.

3.3.5 Human recombinant CES2 oligomerization

The purified human recombinant CES2-10xHis was further evaluated by an in-gel activity assay using native PAGE electrophoresis (Figure 3.5a). To perform this experiment, commercially available CES1 was used as a molecular weight indicator since it is active towards the substrate used. This esterase is active in the trimeric form, but other oligomers are also detected (Figure 3.5a).

CES2-10xHis shows a lighter active band, which should correspond to the monomeric form described, as well as a heavier, less intense form. To our best knowledge, this is the first evidence that hCES2 can be active in structural forms other than monomers. Native PAGE electrophoresis, followed by Western blot, indicates that in 50 ng of CES2-10xHis, different weight oligomers are present (Figure 3.5b). Apparently, the lighter oligomers correspond to the active oligomeric form visualized in the in-gel activity assay.

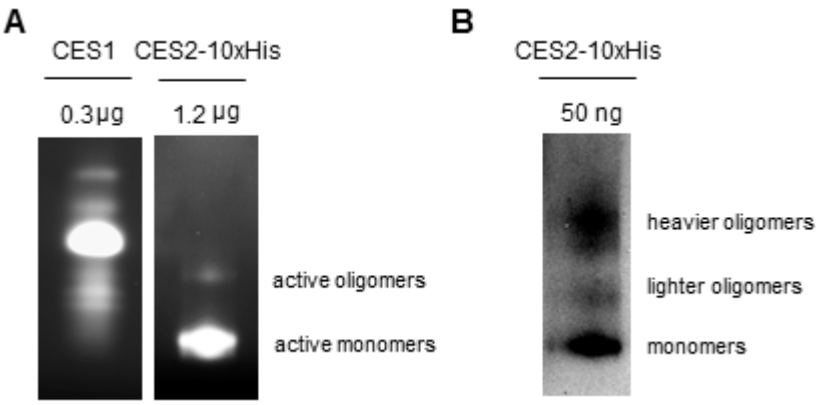

Figure 3.5 Purified CES2-10xHis activity analysis under native conditions. a) *Native PAGE electrophoresis followed by in-gel activity staining with 4-MUBA; 0.3 µg of commercial porcine CES1 (CES1 trimers—180 kDa) and 1.2 µg of human recombinant CES2-10xHis were used. Human carboxylesterase 2 monomers (62 kDa) and active oligomers are shown.* ***b)*** *Native Western blot of 50 ng of CES2-10xHis. Goat anti-CES2 and HRP-conjugated antibodies were used. Carboxylesterase 2 monomers as well as light and heavier oligomers are shown.*

However, the heavier oligomers, visible by Western blot, could not be detected in the activity assay. Using ten-fold less protein amount, only the monomeric form of hCES2 was identified (data not shown). SDS-PAGE electrophoresis performed without reducing and denaturing the samples shows again two types of hCES2 forms (Figure 3.6a). These are also detected, to a lesser extent under denaturing conditions (Figure 3.6b), when SDS-PAGE was performed using denatured and reduced samples, which is probably due to incomplete denaturation of the proteins.

As the oligomeric forms decrease under reducing conditions, the two pairs of cysteines involved in putative disulfide bonds (Pindel et al. 1997) should be involved in the oligomerization. The same results were obtained with other commercially available anti-CES2 antibody, which recognizes a different region of the protein (data not shown).

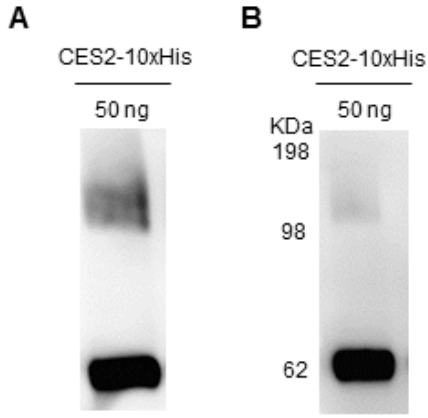

Figure 3.6 Purified CES2-10xHis characterisation by Western blot analysis after SDS-PAGE performed under non-denaturing (a) and denaturing conditions (b)*. Fifty nanograms of CES2-10xHis was used. Carboxylesterase 2 monomers and oligomers are shown. Goat anti-CES2 and HRP-conjugated antibodies were used.*

In all cases, the detection of CES2-10xHis oligomers was hampered at lower protein concentrations (5 ng), which may indicate that these forms were less abundant compared to the monomeric forms. This may also explain why oligomeric active forms of hCES2 were not previously reported. The 10xHis tag present in the C-terminus region of the purified protein is not responsible for the appearance of hCES2 oligomers as these forms were also detected in cell extracts overexpressing hCES2 without the tag (data not shown).

3.4 Discussion

Carboxylesterases have been studied for several years, with the majority of these studies dedicated to mammalian CESs (Takai et al. 1997; Satoh and Hosokawa 2006). It is a very active research field where novel findings continuously emerge. Critical examples are the demonstration of CES absence from human sera (Li et al. 2005) and cocaine hydrolysis exclusively by CES2 and not by CES1 (Hatfield et al. 2010).

To further characterise CESs, particularly hCES2, the present work describes a production process through which hCES2 can be secreted in overexpressing human cells by the addition of an in-frame C-terminal His tag, thus enabling a simple IMAC

purification strategy. Recombinant active CES expression, already reported for several mammalian CESs, in different *in vitro* cell types is mainly performed intracellularly (Morton and Potter 2000; Xie et al. 2002; Sun et al. 2004; Schiel et al. 2007). The secretion of intracellular CESs has been previously described in several cell types and organisms (Morton and Potter 2000). In these, secretion was achieved by one or more of the following methods: addition of a signal sequence in the N-terminus region of the protein (Hermann et al. 2008; Oosterhoff et al. 2002), deletion of the four HTEL C-terminal amino acid residues (Morton and Potter 2000; Hermann et al. 2008; Oosterhoff et al. 2002; Wadkins et al. 2005; Hatfield et al. 2010), and modification of this C-terminal peptide (Robbi and Beaufay 1991, 1992). The secretion of histidine-tagged human CESs, namely hCES1, hCES2, and hCES3, has been previously shown, in insect cells (Williams et al. 2008) by deletion of the KDEL retaining motif.

This work shows that secretion can occur without the deletion or modification of these residues, and we hypothesise that the addition of a 10xHis tag to the C-terminus of hCES2 masks the HTEL motif hampering its binding and retention inside the ER. Secretion of CES2-10xHis due to its overexpression and inability of the cell to cope with large protein amounts was ruled out, since the transfection of HEK-293T cells with a similar expression vector lacking only the 10xHis tag did not lead to the secretion of the protein (Figure 3.1a). Additionally, secretion of hCES2 is not due to cell lysis since, as soon as 24 hpt hCES2 specific activity can be detected in the supernatant of pCI-neo-CES2-10xHis transiently transfected HEK-293T cells but not in the one from pCI-neo-CES2 transiently transfected cells (Figure 3.1a, b). Moreover, addition of brefeldin A, a potent inhibitor of the classical secretory system (Nickel 2010), completely abolishes the secretion of CES2-10xHis to the culture media (Figure 3.3). These results fully support our hypothesis that the deletion of the KDEL retaining motif is not necessary to promote the secretion of the protein and the in-frame His tag located immediately after this sequence is sufficient to induce its secretion. Additionally, one can assume that CES2-10xHis, despite its secretion, was properly

folded and post-translationally modified as it was entering the ER. In fact, purified CES2-10xHis was shown to be sensitive to PNGase F digestion (Figure 3.4b), meaning that the protein was post-translationally modified. Native hCES2 is known to be sensitive to Endo H digestion (Pindel et al. 1997). Since purified CES2-10xHis was secreted, further glycosylation modifications were expected in the Golgi apparatus and explains why the secreted protein bears complex-type glycosylation and not high-mannose type. This glycosylation pattern has previously been shown in rat carboxylesterases (Yan et al. 1995). It was observed that both liver microsomal and secreted forms existed, with the intracellular form sensitive to Endo H digestion and the secreted form sensitive to PNGase F. Additionally, the kinetic parameters (K_M and k_{cat}) for the human recombinant CES2 were determined. K_M is in agreement with the one reported for native human liver CES2 (Pindel et al. 1997) indicating that the recombinant enzyme has similar substrate binding behaviour. Purified recombinant CES2 shows lower catalytic efficiency which may result from differences in assay conditions. Nonetheless, further studies to confirm that the presence of the 10xHis tag or that the different glycosylation pattern are not affecting the activity of the enzyme should be performed in the future.

The levels of intracellular esterase activity both in pCI-neo-CES2-10xHis and pCI-neo-CES2 transiently transfected cells showed an increase throughout time, from 24 to 96 hpt (data not shown). However, the ability of HEK-293T cells to secrete CES2-10xHis led to a dramatic increase in protein productivity since, as soon as 24 hpt, 80% of CES activity was detected extracellularly and only 20% was detected inside the cells (Figure 3.1a). This four-fold increase was only possible due to the secretion of the protein. The production of human recombinant CES2, in a soluble form was boosted to higher levels (50 $mg.L^{-1}$). This is an important aspect since one of the main drawbacks of recombinant protein expression in mammalian cells is the low yields when compared to other expression systems (Morton and Potter 2000; Junge et al. 2008; Schmidt 2004; Zhao et al. 2011). The levels of CES2-10xHis activity show a clear

increase throughout time as the activity in the extracellular supernatant is more than 15-fold higher at 96 than at 24 hpt (Figure 3.1b). This result is easily explained by the stability results of hCES2: the removal of the cells 24 hpt showed no evident decrease in its activity from 48 to 96 hpt (Figure 3.2c).

To our best knowledge, the secretion process of a hCES, namely hCES2, to a serum-free media by simple addition of an in-frame C-terminally localised His tag without the deletion of the HTEL motif in human cells was not previously reported or characterised. This optimised production strategy enabled not only keeping all the characteristics of the protein as no deletion in the gene sequence was performed, but also to perform the expression of the protein as close as possible to its native state, using a human cell line. The majority of the reported CESs purification processes either for the purification of recombinant CESs (Sun et al. 2004) or the purification of native CESs (Beaufay et al. 1974; Morgan et al. 1994; Humerickhouse et al. 2000) involve several highly time-consuming steps. For CES2-10xHis purification, affinity chromatography was chosen. Previous reports of CES purification produced using insect cells showed a huge range of protein yields and purities (Sun et al. 2004; Wadkins et al. 2005; Morton and Potter 2000). The most recently published results for the purification of soluble recombinant CES2 produced in insect cells report a 9% yield of active protein (Hatfield et al. 2010). The obtained yield in the purification strategy followed in the presented work for the purification of soluble CES2-10xHis from HEK-293T cell supernatant is in close agreement with these previously reported yields where a multi-step purification strategy was used. However, in the present work, by applying an IMAC purification strategy, only one purification step was involved.

The establishment of a process for human recombinant CES2 production from human cells enabled further study of this protein. The results presented in this paper point to the existence of active hCES2 in forms other than the 60 kDa monomers

(Figure 3.5a). Moreover, the data also suggests that hCES2 is able to form heavier oligomers. These, however, are only visible by Western blots (Figure 3.5b) and not by in-gel activity assays, which may indicate they are inactive. It was previously reported that esterases, such as esterase D, can form active dimers of 35 kDa monomers (Okada and Wakabayashi 1988); also hCES1 is active as trimers, monomers, and dimers with the hexamers being inactive (Takai et al. 1997; Bencharit et al. 2003; Crow et al. 2007). The ability of carboxylesterase 2 to form organised structures and be active in forms other than monomers is thus not an unusual property and may point for a broader CES shared property. In fact, homology modelling prediction of hCES2 structure based on hCES1 has highlighted the presence of the Z-site region, which modulates hCES1 trimer–hexamer equilibrium, in the C-terminus of hCES2 (Vistoli et al. 2010).

The heavier hCES2 oligomers, visible under native PAGE conditions, are more abundant under non-denaturing conditions (Figure 3.6a) in comparison to denaturing conditions (Figure 3.6b), as expected. In SDS-PAGE electrophoresis performed under non-denaturing conditions, the samples were still in contact with destabilising agents during electrophoresis. Under truly denaturing conditions, the presence of the heavier oligomers may be simply due to incomplete denaturing conditions. Our hypothesis is that hCES2 monomers can associate and form more complex hCES2 structures, depending on the conditions that surround the protein. To further characterise the observed oligomers, size exclusion chromatography would be a good approach in future studies.

The possible formation of very large aggregates, or multimers of more than 500 kDa, was previously mentioned for CESs, but no information concerning those aggregates, their structure, or their activities was reported (Morton and Potter 2000). In the present work, the existence of larger aggregates could not be confirmed by native PAGE and Western blot due to limitations in the protein separation range.

However, no hCES2 activity was detected in the flow-through during the protein purification process. Our hypothesis is that hCES2 may be forming oligomers and/or larger aggregates and thus masking the C-terminal His tag. This aggregation will lead to the formation of inactive hCES2, thereby reducing the obtained yields.

The possibility of forming inactive oligomers may also account for the observed decrease in hCES2 activity in the extracellular supernatant upon the removal of the cells 24 hpt (Figure 3.2c). The observed 20% decrease from 24 to 48 hpt may be due to hCES2 aggregation. The hypothesis that the 10xHis tag could somehow cause the formation of the oligomers was ruled out as they are also found in extracts of untagged hCES2 overexpressing cells (data not shown). These new reported findings challenge the current knowledge about hCES2 and raise several fundamental questions about the structure and activity of this important enzyme. The answer to puzzling questions such as the existence and relevance of these heavier structures in the organism will be crucial and successfully addressed through a multidisciplinary approach where the knowledge of CES2 structure will be the key.

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3.6 References

Beaufay H, Amar-Costesec A, Feytmans E, Thinès-Sempoux D, Wibo M, Robbi M, Berthet J (1974) Analytical study of microsomes and isolated subcellular membranes from rat liver. I Biochemical methods. *J Cell Biol* 61: 188–200.

Bencharit S, Morton CL, Xue Y, Potter PM, Redinbo MR (2003) Structural basis of heroin and cocaine metabolism by a promiscuous human drug-processing enzyme. *Nat Struct Biol* 10: 349–356.

Bornhorst JA, Falke JJ (2003) Purification of proteins using polyhistidine affinity tags. *Methods Enzymol* 326: 245–254.

Crow JA, Borazjani A, Potter PM, Ross MK (2007) Hydrolysis of pyrethroids by human and rat tissues: examination of intestinal, liver and serum carboxylesterases. *Toxicol Appl Pharmacol* 221: 1–12.

Fleming CD, Bencharit S, Edwards CC, Hyatt JL, Tsurkan L, Bai F, Fraga C, Morton CL, Howard-Williams EL, Potter PM, Redinbo MR (2005) Structural insights into drug processing by human carboxylesterase 1: tamoxifen, mevastatin, and inhibition by benzyl. *J Mol Biol* 352: 165–177.

Hatfield MJ, Tsurkan L, Hyatt JL, Yu X, Edwards CC, Hicks LD, Wadkins RM, Potter PM (2010) Biochemical and molecular analysis of carboxylesterase-mediated hydrolysis of cocaine and heroin. *Br J Pharmacol* 160: 1916–1928.

Hermann M, Kietzmann MU, Ivancic M, Zenzmaier C, Luiten RG, Skranc W, Wubbolts M, Winkler M, Birner-Gruenberger R, Pichler H, Schwab H (2008) Alternative pig liver esterase (APLE)—cloning, identification and functional expression in *Pichia pastoris* of a versatile new biocatalyst. *J Biotechnol* 133: 301–310.

Hicks LD, Hyatt JL, Stoddard S, Tsurkan L, Edwards CC, Wadkins RM, Potter PM (2009) Improved, selective, human intestinal carboxylesterase inhibitors designed to modulate 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (Irinotecan; CPT-11). *J Med Chem* 52: 3742–3752.

Holmes RS, Cox LA, VandeBerg JL (2009) Horse carboxylesterases: evidence of six CES1 and four families of CES genes on chromosome 3. *Comp Biochem Physiol Part D Genomics Proteomics* 4: 54–65.

Holmes RS, Wright MW, Laulederkind SJ, Cox LA, Hosokawa M, Imai T, Ishibashi S, Lehner R, Miyazaki M, Perkins EJ, Potter PM, Redinbo MR, Robert J, Satoh T, Yamashita T, Yan B, Yokoi T, Zechner R, Maltais LJ (2010) Recommended nomenclature for five mammalian carboxylesterase gene families: human, mouse and rate genes and proteins. *Mamm Genome* 21: 427–441.

Humerickhouse R, Lohrbach K, Li L, Bosron WF, Dolan ME (2000) Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. *Cancer Res* 60: 1189–1192.

Junge F, Schneider B, Reckel S, Schwarz D, Dötsch V, Bernhard F (2008) Large-scale production of functional membrane proteins. *Cell Mol Life Sci* 65: 1729–1755

Lamego J, Coroadinha AS, Simplício AL (2011) Detection and quantification of carboxylesterase 2 activity by capillary electrophoresis. *Anal Chem* 83: 881–887.

Lange S, Musidlowska A, Schmidt-Dannert C, Schmitt J, Bornscheuer UT (2001) Cloning, functional expression, and characterization of recombinant pig liver esterase. *Chembiochem* 2: 576–582.

Li B, Sedlacek M, Manoharan I, Boopathy R, Duysen EG, Masson P, Lockridge O (2005) Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. *Biochem Pharmacol* 70: 1673–1684.

Liederer BM, Borchardt RT (2006) Enzymes involved in the bioconversion of ester-based prodrugs. *J Pharm Sci* 95: 1177–1195.

Morgan EW, Yan B, Greenway D, Petersen DR, Parkinson A (1994) Purification and characterization of two rat liver microsomal carboxylesterases (hydrolase A and B). *Arch Biochem Biophys* 315: 495–512.

Morton CL, Potter PM (2000) Comparison of *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Spodoptera frugiperda*, and COS7 cells for recombinant gene expression. Application to a rabbit liver carboxylesterase. *Mol Biotechnol* 16: 193–202.

Nickel W (2010) Pathways of unconventional protein secretion. *Curr Opin Biotechnol* 21: 621–626.

Okada Y, Wakabayashi K (1988) Purification and characterization of esterases D-1 and D-2 from human enterocytes. *Arch Biochem Biophys* 263: 130–136.

Oosterhoff D, PinedoHM, van der Meulen IH, deGraaf M, Sone T, Kruyt FA, van BeusechemVW, Haisma HJ, GerritsenWR (2002) Secreted and tumour targeted human carboxylesterase for activation of irinotecan. *Br J Cancer* 87: 659–664.

Pham PL, Kamen A, Durocher Y (2006) Large-scale transfection of mammalian cells for the fast production of recombinant protein. *Mol Biotechnol* 34: 225–237.

Pindel EV, Kedishvili NY, Abraham TL, Brzezinski MR, Zhang J, Dean RA, Bosron WF (1997) Purification and cloning of a broad substrate specificity human liver carboxylesterase that catalyzes the hydrolysis of cocaine and heroin. *J Biol Chem* 272: 14769–14775.

Potter PM, Wolverton JS, Morton CL, Wierdl M, Danks MK (1998) Cellular localization domains of a rabbit and a human carboxylesterase: influence on irinotecan (CPT-11) metabolism by the rabbit enzyme. *Cancer Res* 58: 3627–3632.

Robbi M, Beaufay H (1991) The COOH terminus of several liver carboxylesterases targets these enzymes in the lumen of the endoplasmic reticulum. *Biochem Pharmacol* 71: 657–669.

Robbi M, Beaufay H (1992) Topogenesis of carboxylesterases: a rat liver isoenzyme ending in –HTEHT–COOH is a secreted protein. *Biochem Biophys Res Commun* 183: 836–841.

Ross MK, Borazjani A (2007) Enzymatic activity of human carboxylesterases. *Curr Protoc Toxicol* 33: 4.24.1–4.24.14.

Ross MK, Borazjani A, Edwards CC, Potter PM (2006) Hydrolytic metabolism of pyrethroids by human and other mammalian carboxylesterases. *Biochem Pharmacol* 71: 657–669.

Satoh T, Hosokawa M (2006) Structure, function and regulation of carboxylesterases. *Chem Biol Interact* 162: 195–211.

Schiel MA, Green SL, Davis WI, Sanghani PC, Bosron WF, Sanghani SP (2007) Expression and characterization of a human carboxylesterase 2 splice variant. *J Pharmacol Exp Ther* 323: 94–101.

Schmidt FR (2004) Recombinant expression systems in the pharmaceutical industry. *Appl Microbiol Biotechnol* 65: 363–372.

Schwer H, Langmann T, Daiq R, Becker A, Aslanidis C, Schmitz G (1997) Molecular cloning and characterization of a novel putative carboxylesterase, present in human intestine and liver. *Biochem Biophys Res Commun* 233: 117–120.

Sun Z, Murry DJ, Sanghani SP, Davis WI, Kedishvili NZ, Zou Q, Hurley TD, Bosron WF (2004) Methylphenidate is stereoselectively hydrolysed by human carboxylesterase CES1A1. *J Pharmacol Exp Ther* 310: 469–476.

Takai S, Matsuda A, Usami Y, Adachi T, Sugiyama T, Katagiri Y, Takematsu M, Hirano K (1997) Hydrolytic profile for ester- or amide-linkage by carboxylesterases pI 5.3 and 4.5 from human liver. *Biol Pharm Bull* 20: 869–873.

Tarentino AL, Trimble RM, Maley F (1978) endo-beta-N-Acetylglucosaminidase from Streptomyces plicatus. *Methods Enzymol* 50: 574–580.

Tarentino AL, Plummer TH Jr (1994) Enzymatic deglycosylation of asparagine-linked glycans: purification, properties, and specificity of oligosaccharide-cleaving enzymes from *Flavobacterium meningosepticum*. *Methods Enzymol* 230: 44–57.

Tyminski E, Leroy S, Terada K, Finkelstein DM, Hyatt JL, Danks MK, Potter PM, Saeki Y, Chiocca EA (2005) Brain tumor oncolysis with replication-conditional herpes simplex virus type1 expressing the prodrug-activating genes, CYP2B1 and secreted human intestinal carboxylesterase, in combination with cyclophosphamide and irinotecan. *Cancer Res* 65: 6850–6857.

Uchino J, Takayama K, Harada A, Sone T, Harada T, Curiel DT, Kuroki M, Nakanishi Y (2008) Tumor targeting carboxylesterase fused with anti-CEA scFv improve anticancer effect with a less toxic dose of irinotecan. *Cancer Gene Ther* 15: 94–100.

Vistoli G, Pedretti A, Mazzolari A, Testa B (2010) Homology modeling and metabolism prediction of human carboxylesterase-2 using docking analysis by GriDock: a parallelized tool based on Auto-Dock 4.0. *J Comput Aided Mol Des* 24: 771–787.

Wadkins RM, Hyatt JL, Wei X, Yoon KJ, Wierdl M, Edwards CC, Morton CL, Obenauer JC, Damodaran K, Beroza P, Danks MK, Potter PM (2005) Identification and characterization of novel benzyl (diphenylethane-1,2-dione) analogue as inhibitors of mammalian carboxylesterases. *J Med Chem* 48: 2906–2915.

Waugh DS (2005) Making the most of affinity tags. *Trends Biotechnol* 23: 316–320.

Williams ET, Ehsani ME, Wang X, Wang H, Qian YW, Wrighton SA, Perkins EJ (2008) Effect of buffer components and carrier solvents on in vitro activity of recombinant human carboxylesterases. *J Pharmacol Toxicol Methods* 57: 138–144.

Williams ET, Bacon JA, Bender DM, Lowinger JJ, Guo WK, Eshani ME, Wang X, Wang H, Qian YW, Ruterbories KJ, Wrighton SA, Perkins EJ (2011) Characterization of the expression and activity of carboxylesterases 1 and 2 from the beagle dog, cynomolgus monkey, and human. *Drug Metab Dispos* 39: 2305–2313.

Xie M, Yang D, Liu L, Xue B, Yan B (2002) Human and rodent carboxylesterases: immunorelatedness, overlapping substrate specificity, differential sensitivity to serine enzyme inhibitors, and tumor-related expression. *Drug Metab Dispos* 30: 541–547.

Yan B, Yang D, Bullock P, Parkinson A (1995) Rat serum carboxylesterase. Cloning, expression, regulation, and evidence of secretion from liver. *J Biol Chem* 270: 19128–19134.

Zhao Y, Bishop B, Clay JE, Lu W, Jones M, Daenke S, Siebold C, Stuart DI, Jones EY, Aricescu AR (2011) Automation of large scale transient protein expression in mammalian cells. *J Struct Biol* 175: 209–215.

SECTION 4

Development of a Caco-2 cell line expressing human carboxylesterase 2

Adapted from:

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and

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Abstract

The evaluation of systemic toxicity by xenobiotics frequently includes metabolic studies at the hepatocyte level. Still, ingested xenobiotics find in human intestine the first challenge to reach the entire organism. The study of the toxicological effects of xenobiotics *in vitro* must also account for their fate by this first barrier. Namely it is important to find if xenobiotics permeate and are metabolised in human enterocytes. Human carboxylesterase 2 (hCES2) is the main phase I carboxylesterase (CES) in human intestine. However, it is present at low levels in Caco-2 cells, the most widely accepted *in vitro* model for the study of intestinal absorption.

This work describes the development of a new cell line derived from Caco-2 cells with increased hCES2 activity. A population of Caco-2 cells was stably transfected with *hCES2*, showing a more than two-fold increase in the specific hCES2 activity 21 days after seeding. Increases in hCES2 mRNA (two-fold) and protein levels (six-fold) were also observed, in comparison with Caco-2 cell line. The population was cloned and clones with up to six-fold increases in hCES2 activity were obtained. Nonetheless, stability studies over twenty passages showed instability of protein and activity levels.

Contents

4.1 Introduction

Orally delivered drugs must cross the gastrointestinal wall in order to reach their site of action. To improve the intestinal absorption profile, hydrophilic drugs like carboxylic acids or alcohols, may be transformed into lipophilic prodrugs by esterification. Esters are then bioactivated in the body into their active compounds (Erhardt et al. 2010). A remarkable example is the anti-cancer ester prodrug Irinotecan (CPT-11) that is converted by carboxylesterase 2 (CES2) into its active compound SN-38 (Xu et al. 2002).

Intestinal permeability studies may be performed *in vivo*, *in vitro*, *in situ*, *ex vivo* and *in silico*, and their advantages and pitfalls have been extensively reviewed (Cheng et al. 2008; Buckley et al. 2012; Volpe 2010; Geerts et al. 2011). Most models use animals, animal parts, or animal cells, but it has been demonstrated that the differences existing between animals and humans may pose significant constraints to the extrapolation of data obtained with these models (Crow et al. 2007; Williams et al. 2011). *In vitro* permeability systems include both non cellular models, such as the parallel artificial membrane permeability assay (PAMPA; Reis et al. 2010), and cellular models. Several *in vitro* cellular models exist, enabling the determination of intestinal permeability and are reviewed in Simon-Assman et al. 2007. Caco-2, a human colon adenocarcinoma isolated cell line (Fogh et al. 1977), spontaneously differentiates in culture presenting characteristics of human enterocytes (Pinto et al. 1983). Since its characterisation as an intestinal epithelial permeability model (Hidalgo et al. 1989), this model has become one of the most used for drug absorption prediction (Buckley et al. 2012; Hubatsch et al. 2007), widely applied by industry, and accepted by the regulatory authorities.

Despite its popularity, Caco-2 shows some remarkable differences towards human enterocytes such as in the expression levels of certain transporters and the absence of CYP3A4 metabolising enzymes (Balimane and Chong 2005; Ungell 2004).

Moreover, relative expression levels of carboxylesterases (CESs) in Caco-2 cells were shown not to correlate to those found in human enterocytes. RT-PCR analysis of Caco-2, showed high levels of *human carboxylesterase 1* (*hCES1*) in comparison to the lower ones obtained for *human carboxylesterase 2* (*hCES2*; Imai et al. 2005). On the contrary, through in-gel activity staining of human intestinal microsomes (HIM), almost only hCES2 is detected (Imai et al. 2006). Carboxylesterase 2 is thus the main hCES involved in ester metabolism in the human intestine. The fact that its levels are low in Caco-2 cells, renders this model inappropriate for the study of ester-containing compounds metabolised by hCES2 (Imai and Ohura 2010).

Several attempts have been made to ameliorate Caco-2 gaps and improve its permeability predictions. A wide range of strategies have been applied both for the up- and down-regulation of expression. Chemical agents were used in the induction of protein expression, such as 1 alpha, 25-dihydroxyvitamin D3 that increases CYP3A4 expression (Schmiedlin-Ren et al. 2001), and to inhibit protein activity, such as bis-*p*-nitrophenyl phosphate (BNPP) that inhibits the activity of human CESs (hCESs; Ohura et al. 2010). Genetic manipulation of Caco-2 cells has also been attempted not only for the up-regulation of genes but also for their knockdown, such as the case of Caco-2 transduction with shRNA targeting P-glycoprotein (P-gp; Li et al. 2011).

The goal of the work herein described was to increase hCES2 activity levels in Caco-2 cells in order to develop a model more closely resembling enterocytes in terms of the metabolism of ester containing compounds. To our best knowledge, the genetic modification of these cells, envisioning the overexpression of hCES2, was not previously attempted.

When working with Caco-2 cells there is always the hurdle of both intra- and inter-laboratory variability and efforts have been made to understand and minimize these differences (Roth et al. 2012, Natoli et al. 2012; Prieto et al. 2010; Hayeshi et al. 2008; Coecke et al. 2005). Nonetheless, the effect of seeding inocula on hCES2

expression was not previously reported. This work thus starts with a full characterisation of hCES2 activity in Caco-2 cells and the effect of both seeding inocula and passage number followed by the generation and characterisation of a stably transfected cell line with increased levels of hCES2 activity. The evaluation of hCES2 expression stability in the newly generated cell line provides an important contribution to the field of *in vitro* cell models refinement by showing a common problematic hurdle on Caco-2 cells manipulation that appears to be independent of the expressed transgenes, i.e. genes other than *hCES2*.

4.2 Materials and Methods

4.2.1 Materials

Bovine alkaline phosphatase (ALP), 4-Methylumbelliferyl acetate (4-MUBA) (≥98%), 4-methylumbelliferone (4-MUB) (≥98%), 4-methylumbelliferyl phosphate (4-MUP), Trizma hydrochloride (>99%), Trizma base (>99.9%), Tris-buffered saline (p.a.), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) (>99.9%), HEPES (≥99.5%), sodium hydroxide (≥98%) and Triton X-100 (laboratory grade) were from Sigma (St. Louis, U.S.A.). Potassium chloride (≥99.5%) was from Aldrich (Steinheim, Germany). Sodium acetate (≥99.0%) and loperamide hydrochloride (p.a.) were from Fluka (Seelze, Germany). Potassium dihydrogen phosphate (≥99%) and ethanol (99.5%) were from Panreac (Barcelona, Spain). Sodium chloride (≥99.5%), albumin fraction V (≥98.0%), and skim milk (for microbiology) were from Merck (Darmstadt, Germany). Dimethyl sulphoxide (DMSO; 95%) was from Lab-Scan (Dublin, Ireland). Milli-Q water (Direct-Q 3 UV ultrapure water purification system from Millipore; Billerica, U.S.A.) was used for the preparation of all solutions.

4.2.2 Cells and media

Caco-2 cells (ATCC HTB-37), between passage number 26 to 41, were routinely cultured in T-75 flasks (BD Biosciences) using high glucose (4.5 g/L)

Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (*v/v*) fetal bovine serum (FBS) and 1% (*v/v*) non-essential aminoacids (Gibco; Grand Island, U.S.A.), at 37 °C in a humidified atmosphere containing 7.5% (*v/v*) CO_2 in air. Once a week, before reaching confluence, cells were cultured using Dulbecco's phosphate-buffered saline (DPBS) and 0.25% (*w/v*) Trypsin-EDTA (Gibco). Media replacement was performed in alternating days. Cell concentration and viability were determined with a Fuchs–Rosenthal counting chamber (Marienfeld; Lauda-Königshofen, Germany) using 0.1% (*v/v*) trypan blue (Gibco) staining. Cell monolayers were prepared, unless otherwise stated, by seeding 1.04×10^4 cell.cm^{-2} in 100 mm i.d. tissue culture petri plates (Becton Dickinson; Franklin Lakes, U.S.A.) or 6-well tissue culture plates (Becton Dickinson) for protein and RNA extracts, according to the needed extract amounts. For alkaline phosphatase activity determination, 96-well tissue culture plates were used. Media change was performed in alternating days for the first week and every day for the remaining time in culture until 21 days.

4.2.3 Plasmids

Human *CES2* gene (geneID 8824) was cloned in pCI-neo plasmid (Promega; Madison, U.S.A.) using *SalI* and *NotI* restriction endonucleases (New England Biolabs; Ipswich, U.S.A.) as previously described (Section 3; please see Appendices for vector map). The construct in pCI-neo-CES2 plasmid was fully sequenced to verify the integrity of the *hCES2* gene. Production and purification of the expression vector was performed and evaluated according to standard molecular biology techniques.

4.2.4 Caco-2 CES2 cell line establishment

Caco-2 cells were transfected in 6-well tissue culture plates with 20 µg.mL^{-1} pCI-neo-CES2 plasmid using 60 µg.mL^{-1} polycation polyethylenimine (PEI; Polysciences; Eppelheim, Germany) 24 h after seeding at a cell density of 1.04×10^4 cell.cm^{-2}. Selection pressure was started 48 h post-transfection (hpt) using 0.95 mg.mL^{-1} of

G418 antibiotic (Invitrogen). Medium was changed twice during the first week. One week after selection pressure was started, amplification of the selected population was performed and a master cell bank of Caco-2 CES2 population was created.

Cell cloning was performed through the limiting dilution method. Briefly, cells were seeded at 1 cell per well in 96-well tissue culture plates using 50% (*v/v*) conditioned media supplemented with 20% (*v/v*) FBS and 0.48 $mg.mL^{-1}$ of G418 antibiotic. More than 80 clones were picked from single colony wells. Step-wise amplification of each clone was performed, with a gradual increase of available cell culture area. 43 clones survived this process and were appropriately stored and analysed.

4.2.5 Real-time qPCR analysis

Total RNA from Caco-2 cell line and Caco-2 CES2 cell population, at passages 30 and 39, respectively, were extracted at 7, 14, and 21 days after seeding using RNeasy extraction kit (Qiagen; Courtaboeuf, France) according to the manufacturer's instructions. Samples were treated with DNAse. Cells were grown in 6-well tissue culture plates as detailed above and three wells were independently extracted for each cell type at each time point. RNA concentration and purity was determined using NanoDrop 2000c spectrophotometer (Thermo Scientific; Wilmington, U.S.A.).

Messenger RNA was reverse transcribed to cDNA with Transcriptor High Fidelity cDNA synthesis kit (Roche; Mannheim, Germany), using anchored oligo (dT) primer and 2 µg of total RNA per reaction, according to the manufacturer's instructions. Real-time PCR analysis was performed using SYBR Green in a LightCycler 480 System PCR (Roche), according to the manufacturer's instructions. Previously described and tested RT-PCR primers and amplification conditions (Sanghani et al. 2003) for *hCES1*, *hCES2* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH)* genes were used. Negative controls were also run in parallel with the cDNA samples

to detect contamination problems during PCR analysis and/or DNA contamination during RNA extraction. Analysis of relative gene expression of *hCES1* and *hCES2* compared to the housekeeping *GAPDH* gene, previously shown to be invariant from undifferentiated to differentiated stages of Caco-2 cells (Bédrine-Ferran et al. 2004), was performed according to the $2^{-\Delta CT}$ method (Livak et al. 2001).

4.2.6 Western blot analysis

Caco-2 cell extracts were prepared at 7, 14, and 21 days after seeding, as previously reported (Lea et al. 2010), with minor modifications. Briefly, cells grown in 6-well plates were washed with DPBS and rapidly lysed with 200 µL of a solution containing 0.25 mM NaCl, 5 mM EDTA, and 1% (*v/v*) Triton (Korjamo et al. 2006) in 50 mM Tris-HCl pH 8.0. Cell extracts thus prepared were centrifuged at 10,000*g* for 10 min at 4 °C and were stored at −20 °C. Total protein concentration was determined using bicinchoninic acid (BCA) protein assay (Pierce Biotechnology; Rockford, U.S.A.), according to the manufacturer's instructions.

Unless otherwise stated for SDS-PAGE electrophoresis, the same total protein amount (30 µg) was loaded onto NuPAGE 4–12% (*w/v*) bis-tris acrylamide gels (Invitrogen; Carlsbad, U.S.A.). All samples were previously denatured and reduced for 10 min at 70 °C. Electrophoresis was performed in XCell SureLock mini-cell system (Invitrogen) using MOPS SDS running buffer (Invitrogen), according to the manufacturer's instructions. Proteins were transferred from the gels to PVDF (Millipore) membranes and blocked overnight (ON) with 5% (*w/v*) milk in 0.05% (*v/v*) Tween, Tris-buffered saline solution, pH 7.6. Semi-dry transfer was performed using Amersham Hoefer TE 70 transfer unit (GE Healthcare; Little Chalfont, U.K.) according to the manufacturer's protocol.

The following primary antibodies were used: goat anti-hCES2 (1:200) from R&D systems (Minneapolis, U.S.A.); rabbit anti-hCES1 (1:250) from Sigma; and mouse

anti-β actin (1:500) from Abcam (Cambridge, U.S.A.). Secondary antibodies used were: horseradish peroxidase (HRP)-rabbit anti-goat (1:5,000) from Invitrogen; HRP-donkey anti-rabbit (1:20,000) and HRP-sheep anti-mouse (1:25,000) from GE Healthcare. Chemiluminescent detection was performed with ECL Prime (GE Healthcare). Image acquisition was performed with ChemiDoc (Bio-Rad; Hercules, U.S.A.) and non-saturated images were quantified through densitometry with ImageJ open source software (NIH; Bethesda, U.S.A.). Integrated density values were normalised per lane with the ones obtained for β-actin. Each result is shown in relation to Caco-2 at 21 days, which was used as reference in each gel. Each experiment was repeated at least twice to confirm the verified pattern.

4.2.7 Esterase activity determination

Caco-2 cell extracts were performed as described above. The enzymatic activity assays were performed using the substrate 4-MUBA as previously described (Lamego et al. 2012). Briefly, reactions were undertaken in a final reaction volume of 250 µL, at 37 °C under substrate saturation conditions. Unless otherwise stated, the same total protein amount (10 µg) was used in each assay. 90 mM potassium dihydrogen phosphate and 40 mM potassium chloride (pH 7.3) were used as reaction buffer. 4-MUB production was monitored at 350 nm (Pindel et al. 1997) on a SpectraMax 340 plate reader (Molecular Devices; Sunnyvale, U.S.A.). Results of esterase activity are presented as micromoles per minute per milligram of total protein. Mean *t* tests were performed for evaluation of statistically significant differences (p = 0.05) between Caco-2 and Caco-2 CES2 samples.

4.2.8 Alkaline phosphatase activity determination

Alkaline phosphatase activity was measured by the activity towards 4-MUP, as previously described (Malpique et al. 2007). Briefly, in each time point (7, 14, and 21 days), cells from 4 independent wells were washed and incubated with 200 µL of a 1:1 mixture of 1 mM 4-MUP in 20 mM HEPES/NaOH, pH 7.4 and 20 mM HEPES/NaOH, pH 9.0. Light protected incubations were performed for 1.5 h, 20 min, and 8 min for the 7^{th}, 14^{th}, and 21^{st} day, respectively. 75 µL of supernatant was transferred to a 96-well black microplate (Greiner Bio-One; Frickenhausen, Germany). 4-MUB production was monitored at 535 nm (excitation at 360 nm) on a GloMax Multi detection system fluorescence plate reader (Promega). Calibration curves were obtained with ALP in the 0.5 to 20 $\mu g.mL^{-1}$ (0.43 to 17 microUnits) range. Substrate spontaneous hydrolysis was accounted. Results are presented as microUnits per viable cell (one unit is defined as the activity that hydrolyses one µmol of substrate per minute).

4.3 Results

4.3.1 Evaluating hCES2 variability in Caco-2 cells

Several cell and culture-related factors have been shown to influence Caco-2 development and characteristics (Sambuy Y et al. 2005; Prieto et al. 2010; Christensen et al. 2012). Culture medium has shown to influence the expression of different drug transporters and drug metabolising enzymes, including hCES2 (Roth et al. 2012). In this study, the researchers found that *hCES2* mRNA was slightly up-regulated, without statistical significance, when Caco-2 cells were cultured in DMEM (the medium used in the present study) in comparison with minimum essential medium alpha (AMEM). The initial seeding inoculum of Caco-2 cells has been reported to impact not only cell morphology but also the expression of different proteins, including drug transporters and drug metabolising enzymes (Volpe 2008; Natoli et al. 2011; Roth et al. 2012). To our best knowledge, the effect of cell seeding on hCES2 activity was never evaluated.

To study this, Caco-2 cells were cultured at passage 31 with different seeding inocula from $1.0x10^4$ to $1.0x10^5$ cell.cm^{-2} (Figure 4.1) and allowed to differentiate for 21 days before testing, as described in the Materials and Methods. The results on specific esterase activity did not show statistically significant variations (Figure 4.1a). However, in the presence of loperamide, a selective CES2 inhibitor (Williams et al. 2011), variations on hCES2 specific activity were higher, leading to a tendency of increased inhibition percentages with higher seeding densities, more evident at higher starting seeding inocula (Figure 4.1b) but still with no statistical relevance.

Passage number has also been indicated as a biological factor influencing variability and protein expression profiles in Caco-2 cells (Shah et al. 2006; Siissalo et al. 2007). The expression profile of hCES2 (as well as hCES1) was reported to be constant between passages 28 and 59 in different laboratories, based on native PAGE electrophoresis followed by staining for esterase activity (Imai et al. 2005; Imai 2006), but no data was shown. Nonetheless, due to several reports on Caco-2 intra and inter-laboratory variability (Press and Di Grandi 2008; Volpe 2008), hCES2 activity was herein assessed at different passages (Figure 4.2). This step is mandatory to understand the passage range in which it is appropriate to modify these cells, namely, to increase hCES2. Two different seeding inocula were used, $1.0x10^4$ and $2.0x10^4$ cell.cm^{-2}. No statistically significant difference was observed when comparing both passages under different conditions - in the presence and absence of inhibitor and with different starting inocula.

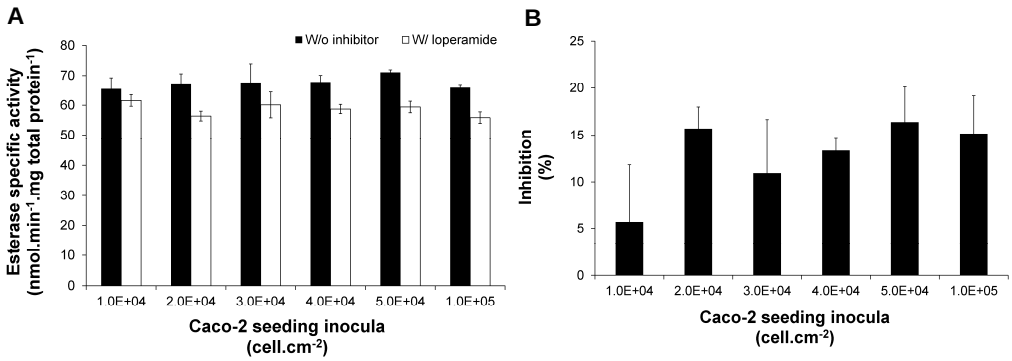

Figure 4.1 Seeding inocula effect on esterase activity. a) *Caco-2 cells, at different starting seeding inocula were tested for esterase specific activity in the absence and presence of loperamide.* ***b)*** *Inhibition percentage was calculated through the difference between the activity in the absence and presence of the inhibitor, as described before (Lamego et al. 2011). Each result represents the average of three independent assays, and error bars represent the standard deviation* ***(a)*** *or the combined standard deviation* ***(b)****.*

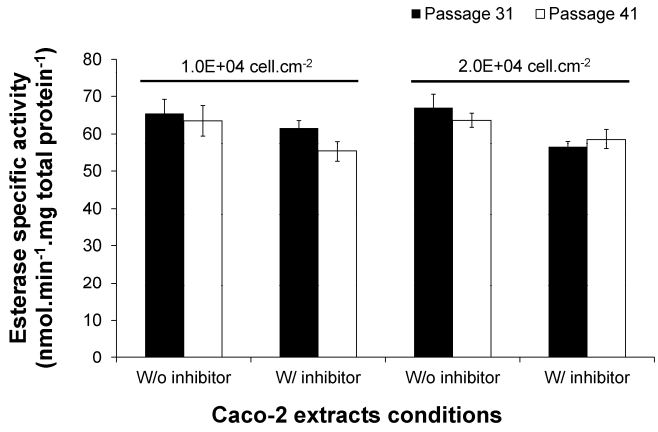

Figure 4.2 Effect of passage number in esterase activity. *Caco-2 cells, in two different passage numbers, were tested for esterase specific activity in the absence and presence of loperamide, at two different starting seeding inocula. Each result represents the average of three independent assays, and error bars represent the standard deviation.*

4.3.2 hCES2 transient expression in Caco-2 cells

To increase hCES2 activity levels, an expression vector was generated using pCI-neo plasmid as a backbone. Caco-2 cells were transiently transfected with pCI-neo-CES2, using two different seeding inocula, $1.0x10^4$ and $5.0x10^4$ cell.cm^{-2}, as described in Materials and Methods. Esterase specific activity was evaluated 48 hpt (72 h after seeding) in the presence and absence of loperamide (Figure 4.3). Inhibition percentage of the transfected samples (Caco-2 CES2), calculated through the difference between the activity in the absence and presence of the inhibitor, as described before (Lamego et al. 2011), was the same, 28 ± 7%, independently of the starting seeding inocula. No inhibition was detected in Caco-2 cells 48 hpt, as expected. The increase in esterase specific activity from parental to transiently transfected cells is thus shown to be due to the increase in hCES2 activity.

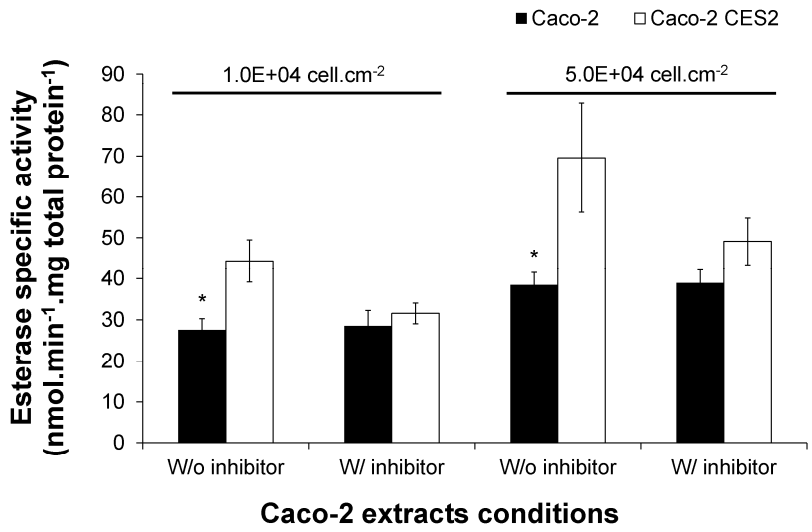

Figure 4.3 Esterase specific activity in Caco-2 transiently transfected cells. *Caco-2 cells, transfected at $1.0x10^4$ and $5.0x10^4$ cell.cm^{-2} seeding inocula were tested for esterase specific activity in the absence and presence of loperamide 48 hpt. Each result represents the average of three independent assays, and error bars represent the standard deviation. A * represents a statistically significant difference (p = 0.05) between Caco-2 and Caco-2 CES2 samples.*

4.3.3 Characterisation of Caco-2 CES2 population

Since pCI-neo-CES2 plasmid bears the *neomycin* resistance gene, geneticin antibiotic (0.95 mg.mL^{-1}) was used as selection agent to obtain a Caco-2 CES2 population that could be propagated. A kill curve using 0.5 to 2 mg.mL^{-1} of geneticin was performed and 0.95 mg.mL^{-1} was chosen as the most effective antibiotic concentration (data not shown). Esterase specific activity was determined in parental Caco-2 and selected Caco-2 CES2 population at different time points (Figure 4.4). Although no statistically significant difference was observed in total esterase specific activity between parental Caco-2 and Caco-2 CES2 population (Figure 4.4a), there was a clear difference in hCES2 specific activity as reflected by an increase in the inhibition by loperamide (Figure 4.4b). A small increase in hCES2 activity, without statistical significance, was detected in Caco-2 cell line throughout the differentiation period. However, a 2.5-fold increase in inhibition percentage was observed from the first to the second week of the differentiation period in Caco-2 CES2 population.

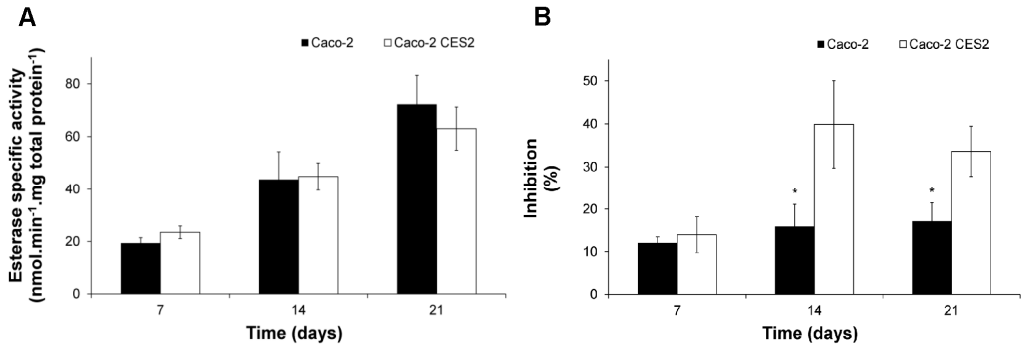

Figure 4.4 Esterase specific activity in Caco-2 CES2 selected population. *Caco-2 (P39) and Caco-2 CES2 (P37) were seeded at 1.0x10^{4} cell.cm^{-2}, in the absence and presence of geneticin, respectively. Esterase and hCES2 specific activities were determined at different time points. Each result represents the average of three independent assays.* ***a)*** *Esterase specific activity determined in the absence of loperamide. Error bars represent the standard deviation of three independent assays.* ***b)*** *hCES2 specific activity showed in terms of inhibition percentage, determined in the presence of loperamide. Error bars represent the combined standard deviation. A * represents a statistically significant difference (p = 0.05) between Caco-2 and Caco-2 CES2 samples.*

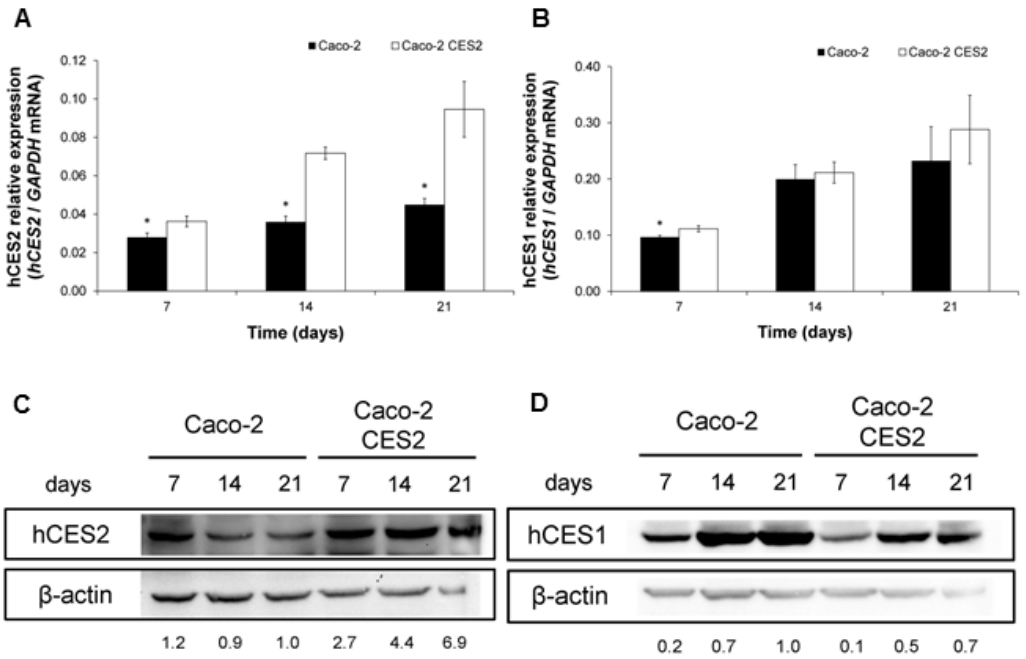

Figure 4.5 Carboxylesterase gene and protein expression in Caco-2 and Caco-2 CES2 cells. a, b) *hCES2 and hCES1 relative expression was evaluated in both Caco-2 cell line and Caco-2 CES2 population, throughout the differentiation period, by qRT-PCR. GAPDH was used for normalisation. Error bars represent the standard deviation. A * represents a statistically significant difference (p = 0.05) between Caco-2 and Caco-2 CES2 samples.* ***c, d)*** *Relative protein expression of hCES2 and hCES1 was evaluated in both cell types, using Caco-2 21 days as reference sample. Integrated density values were normalised per lane towards β-actin.*

Both cell types were also evaluated for protein and gene expression levels (Figure 4.5). Human *CES1* relative mRNA levels increased over culture time in both parental and stably transfected Caco-2 cells and the same increasing pattern was shown for protein. Human *CES2* relative mRNA levels are always significantly higher in Caco-2 CES2 population in comparison to the parental cell line and increase throughout the differentiation period. The increase was, nonetheless, more pronounced in Caco-2 CES2 population since the *hCES2* relative mRNA levels practically doubled from the 7th to the 21st day, whereas in Caco-2 parental cells an approximate 60% increase occurred. By evaluating hCES2 protein levels, a similar pattern was found. Human *CES1* relative mRNA levels were always higher in both

Caco-2 and Caco-2 CES2, compared with *hCES2*. It should be noted the amplified region for *hCES1* (190 base pairs) and *hCES2* (310 base pairs) are different.

The influence of hCES2 expression was also evaluated in the differentiation properties of the transfected population. Alkaline phosphatase activity per viable cell was used as a marker of differentiation. No difference was observed at day 21 between Caco-2 parental cell line and Caco-2 CES2 population (Figure 4.6).

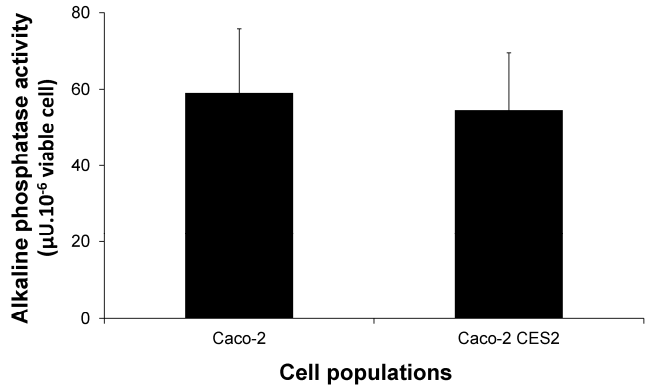

Figure 4.6 Alkaline phosphatase expression in Caco-2 cell line and Caco-2 CES2 population. *ALP activity was measured at day 21, as described in Materials and Methods, and normalised with the number of viable cells. Error bars represent the standard deviation.*

4.3.4 Caco-2 CES2 cell line establishment and expression stability evaluation

For the establishment of a Caco-2 CES2 cell line, 84 single-cell clones were selected through limiting dilution, as described in Materials and Methods. Only 43 grew well enough to be tested. Clones 1 to 14, depicted in Figure 4.7 had higher activity compared with parental Caco-2 cells. The stability of hCES2 expression was tested in the highest expressing cell lines. Figure 4.8 shows the results for clone 14 in a time window of twenty passages, one passage per week. In the first ten passages, passages 41 to 51, the effect of geneticin antibiotic was tested by culturing the cells in the presence and absence of the antibiotic. From passage 51 onwards, no antibiotic was used. Clone 14 showed higher hCES2 specific activity levels than the ones found for

Caco-2 CES2 stably transfected population and this was also reflected in the relative protein expression levels.

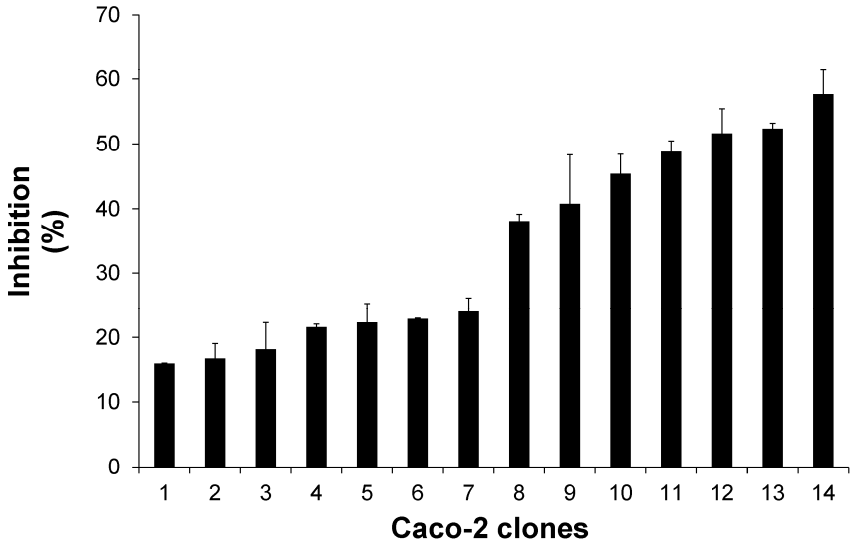

Figure 4.7 hCES2 specific activity in stably transfected clones. *Caco-2 CES2 selected clones were tested at day 21, at passage 41, for esterase specific activity in the absence and presence of loperamide and inhibition percentage was calculated. Each result represents the average of three independent assays, and error bars represent the combined standard deviation.*

However, there is a dramatic decrease in both hCES2 activity and protein levels that is independent of the presence of geneticin. In 10 weeks, the cell line reverts completely to the levels of hCES2 activity detected for Caco-2 parental cell line, not recovering in the following 10 weeks. This same tendency was verified for other clones, confirming the detected pattern: 11 clones were tested at passage number 51, and 3 at passage number 61.

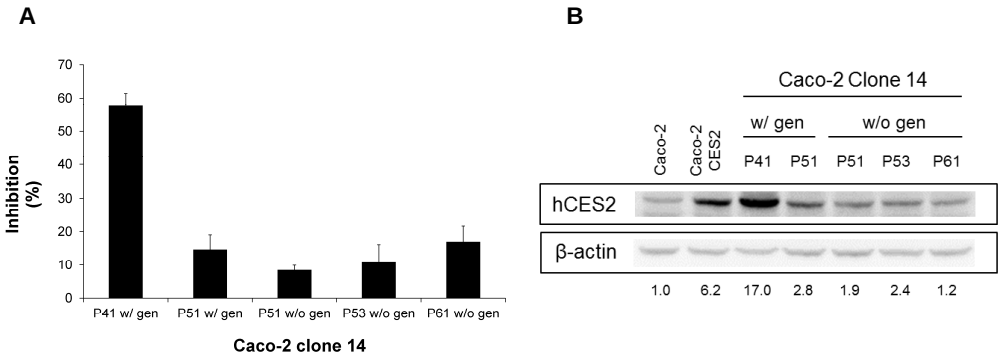

Figure 4.8 hCES2 expression stability evaluation in Caco-2 CES2 cell line. *Caco-2 CES2 cell line was tested, at day 21, at different passages. Caco-2, P39, and Caco-2 CES2, P37, were used as controls.* ***a)*** *Inhibition % calculated through esterase specific activity in the absence and presence of loperamide. Each result represents the average of three independent assays, and error bars represent the combined standard deviation.* ***b)*** *Relative hCES2 expression, using Caco-2 sample as reference. Integrated density values were normalised per lane with β-actin. w/gen – with geneticin; w/o gen – without geneticin.*

4.4 Discussion

As highlighted before, Caco-2, a cell line derived from a human tumour, was reported to bear low levels of hCES2 and high levels of hCES1 expression while evidence showed that hCES2 is expressed to a higher extent than hCES1 in normal colon cells (Imai et al. 2005). This still unanswered question impelled us to overexpress hCES2 in Caco-2 cells and evaluate the stability of the obtained cell line.

The Caco-2 cell line is well known to be sensitive to variations in culture conditions. Seeding density and passage number are some of the factors impacting the expression profiles of some transporters such as the mRNA of *P-gp* (Prieto et al. 2010; Hayeshi et al. 2008).

Human carboxylesterase 2 mRNA levels have been previously shown to increase throughout the differentiation period of Caco-2 cells (Ohura et al. 2010). In this work, we not only confirmed these results but also demonstrated how passage number and seeding inocula influence the activity levels of this enzyme in Caco-2 cells. A tendency for increased hCES2 specific activity, specifically measured in the presence of loperamide, with higher seeding inocula was detected. Nonetheless it fell

within the standard deviation of independently performed experiments, pointing to the high biological variability affecting this system (Figure 4.1). A tendency towards a decrease in hCES2 specific activity at higher passages was detected (Figure 4.2). This may provide an indication of the intrinsic instability of this cell line towards hCES2 expression. Nonetheless, as no statistically significant difference was observed in this 10 cell-passage interval, it provides an appropriate window of time for manipulating Caco-2 cells (Figure 4.2).

Fitted with a deeper knowledge of the system, the next logical step was to genetically modify Caco-2 cells. There are several gene delivery vehicle-DNA complexes described in the literature. From these, PEI and lipofectamine are the two chemical transfection agents most commonly used (O' Neill et al. 2011) and have been previously applied in Caco-2 cells (Gautrey et al. 2011; Korjamo et al. 2005). PEI, being less expensive, was the chosen approach, demonstrated to be suitable for hCES2 transient overexpression as the esterase specific activity levels were increased 48 hpt (Figure 4.3). The increase in hCES2 specific activity was shown to be independent of the seeding inocula used. Curiously, the obtained hCES2 activity 48 hpt was higher than the maximum detected in Caco-2 parental cells after 3 weeks in culture (Figures 4.1b and 4.3), suggesting the ability of this cell line to cope with increased levels of active hCES2. A stably transfected Caco-2 CES2 population was established, with increased hCES2 specific activity (Figure 4.4b). Surprisingly, no statistically significant difference was observed in total esterase activity between parental Caco-2 and Caco-2 CES2 population (Figure 4.4a). This may be due to substrate competition as both hCES1 and hCES2 are known to hydrolyse 4-MUBA.

Through limiting dilution, it was possible to select a Caco-2 CES2 cell line. Caco-2 CES2 population heterogeneity was revealed in the diversity of hCES2 activity in the 14 clones depicted in Figure 4.7. As previously reported, protein expression levels of hCES1 and hCES2 do not correlate with their enzymatic specific activities

(Ross and Crow 2007). In this work additional evidence is provided as there is not a proportional correlation between activity (Figure 4.4b), mRNA (Figure 4.5a, b), and protein expression (Figure 4.5c, d) of both hCESs. These differences may rely in the technical constraints inherent to each analytical technique such as the amplification efficiency in qRT-PCR, related, for instance with the size of the amplified region which is different for hCES1 and hCES2, or the different antibody binding affinities in Western blot. In order to avoid misleading results, the screening of Caco-2 CES2 expressing clones was based on the assessment of hCES2 specific activity and later complemented with protein expression studies.

To evaluate the feasibility of the usage of this newly generated cell line, with increased levels of active hCES2, as a stable and able to be propagated platform for drug screening, expression stability tests were performed by culturing the cells for increased periods of time. A severe loss of hCES2 activity, reaching basal population levels in 10 weeks was shown not to correlate with the presence of the selection pressure agent geneticin (Figure 4.8a). This phenotype, verified for several of the selected clones, was not reverted in the following ten weeks of culture in the absence of the antibiotic. We have further discovered that the decreased hCES2 activity was reflected in the levels of protein expression (Figure 4.8b).

Stable expression of hCES2 was previously reported to be possible through a genetic engineering strategy almost identical to the one followed in the present study, but using Chinese Hamster Fibroblast cell line (VT79; Imai et al. 2006). This evidence suggests that it should be possible to stably express recombinant hCES2 in mammalian cells, under the control of the CMV promoter, using chemical transfection techniques combined with selection with geneticin selection. However, it was previously reported not to be possible to stably increase hCES2 levels in COS-7 electroporated cells following a similar approach (Wierdl et al. 2008).

Expression of proteins was previously shown to be possible in Caco-2 cells. Examples are the increase in Glutathione peroxidase 4 (GPx4; Gautrey et al. 2011) and the decrease in P-gp levels (Hilgendorf et al. 2004). Using the same genetic engineering approach, i.e. the same expression vector backbone, including the same promoter, as well as the same type of selection pressure of the one chosen in the present study, it was previously reported to be possible to increase the levels of membrane dipeptidase (MDP; Pang et al. 2004). However, no data showing the stability of the obtained cells throughout time in culture, in any of these cases, was shown.

The problem of Caco-2 modification has been previously highlighted in terms of the difficulty of increasing CYP expression (Korjamo et al. 2006). Combining the results presented herein with data previously reported, and exemplified in table 4.1, there is evidence of an overall problem in stable Caco-2 modification. A possible common mechanism through which Caco-2 effectively represses the expression of exogenous genes may thus be envisioned. This seems to be independent of the transgene, the transfection method and the selection pressure used, as the same pattern was previously reported for CYP3A4 and CYP2A6, electroporated and selected with hygromycin B (Crespi et al. 1996).

We have confirmed that this repression does not occur at the enzymatic activity level as a decrease in protein expression level was also detected. Since a clear response of *CES2* mRNA during the differentiation period in Caco-2 CES2 stably transfected population occurs we hypothesise that this may be the level of regulation of exogenous hCES2 expression, may it be through promoter inhibition or decreased mRNA stability. It cannot be excluded, however, that other repression mechanisms may also exist. It was previously shown a direct link between transcript inactivation and epigenetic modification in stably transfected cell lines gradually loosing transgene expression.

Table 4.1 Non-differentiated Caco-2 stable transfection

Gene/ Protein	Up / down reg	Promoter type	Plasmid type	Transfection method	Selecting agent	Expression stability	Reference
Glutathione peroxidase 4 (GPx4)	Up	Viral: CMV	pCDNA3.1	Chemical: Lipofectamine 2000	zeocin antibiotic	Clones selected; no report towards expression stability throughout passaging	Gautry et al. 2011
Membrane dipeptidase	Up	Viral: CMV	pCIneo	Physical: electroporation	neomycin antibiotic	Selection performed; no report towards expression stability	Pang et al. 2004
P-glycoprotein (P-gp)	Down	Viral: SV40	pEUK-C1; pMAMneo	Physical: electroporation	G418 antibiotic	Clones selected; no report towards expression stability throughout passaging	Hilgendorf et al. 2004
Human pregnane X receptor (hPXR)	Up	Viral: CMV	pCIneo	Chemical: polycation polyethylenimine (PEI)	G418 antibiotic	Clones selected; reported to be stable for 15 passages; results are not shown	Korjamo et al. 2005
Murine constitutive androstane receptor (mCAR)	Up	Viral: CMV	pCIneo	Chemical: polycation polyethylenimine (PEI)	G418 antibiotic	Clones selected; reported to be stable for 12 passages; results are not shown	Korjamo et al. 2005
P-gp	Up	Viral: CMV	pCIneo	Chemical: polycation polyethylenimine (PEI)	G418 antibiotic	Clones selected; reported to be stable for 6 passages; results are not shown	Korjamo et al. 2005
Caudal-related homeodomain protein 2 (CDX2)	Up	Viral: CMV	pTarget	Chemical: Lipofectamine 2000	G418 antibiotic	Clones selected; no report towards expression stability throughout passaging	Kusano et al. 2012
CYP3A4, CYP3A6	Up	Viral: CMV	p220.2	Physical: electroporation	Hygromycin B antibiotic	CYP3A4 clones selected; activity reported to decrease five-fold in five passages.	Crespi et al. 1996
P-gp	Down	Human: Modified U6 promoter	Lentiviral vectors with shRNA for P-gp (Sigma)	Transduction: Lentivirus	Puromycin antibiotic	Clones selected for 5 passages	Li et al. 2011

In fact, it was shown that transcript inactivation is followed by promoter DNA methylation and histone H3 K9 dimethylation, being the early events of transgene silencing the acetylation of histones and their methylation at histone H3 K4 (Mutskov and Felsenfeld, 2004).

Several techniques may help understand and overcome the reasons behind the repression of hCES2, in particular, and transfected genes, in general, in Caco-2 cells. The application of chemical inducers to restore the repressed levels will, for instance, influence the expression of other proteins and/or Caco-2 morphology. As our goal was to increase hCES2, interfering as little as possible with the parental cell environment, it may be concluded that Caco-2 manipulation by transient transfection is the fastest and most suitable approach to avoid the intrinsic regulation mechanisms of this cell line. Different promoters, both viral and cellular may then be tested. For those cases where it is not possible to cope with the variability associated with the transient transfection approach, the establishment of a larger and well characterised master cell bank may constitute a valid approach, as previously suggested (Crespi et al. 1996). In this case, geneticin should be the elected antibiotic, instead of others such as hygromycin B. Opposing to what was described to happen with hygromycin B (Crespi et al. 1996; Rodolosse et al. 1998), no defects in cell adherence, monolayer integrity or cell morphology was detected using geneticin.

In the future, more innovative approaches in Caco-2 manipulation may contribute to overcome its problematic manipulation with the potential to constitute a more generalized solution such as the targeted insertion of genes into pre-selected highly transcribed regions of the genome. Further improvements to Caco-2 may then be envisioned, such as *hCES1* down-regulation, to more closely resemble human enterocytes.

A detailed comparison of the newly developed Caco-2 CES2 cell line will be needed, being advisable to use 20 passively absorbed drugs, representing a range

from low to high intestinal absorption (Volpe et al. 2007), to adequately demonstrate its suitability for testing a drug's permeability and all the other currently performed tests using Caco-2 cells.

4.5 Conclusions

The work herein presented describes the efforts towards the improvement of currently available *in vitro* tools highly used in research and development through the establishment of a new cell line derived from Caco-2 cells with increased hCES2 activity levels. The new cell line, better reflecting human intestinal metabolism towards hCES2 metabolised esters, retains the polarisation and differentiation capabilities of the parental cell line, thus having the potential to become, in the future and upon complete validation with reference compounds, a useful tool for coupling the study of intestinal absorption with intestinal metabolism, especially of ester containing drugs and prodrugs.

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4.7 References

Balimane PV, Chong S (2005) Cell culture-based models for intestinal permeability: a critique. *Drug Discov Today* 10: 335-343.

Bédrine-Ferran H, Le Meur N, Gicquel I, Le Cunff M, Soriano N, Guisle I, Mottier S, Monnier A, Teusan R, Fergelot P, Le Gall JY, Léger J, Mosser J (2004) Transcriptome variations in human Caco-2 cells: a model for enterocyte differentiation and its link to iron absorption. *Genomics* 83: 772-789.

Buckley ST, Fischer SM, Fricker G, Brandl M (2012) In vitro models to evaluate the permeability of poorly soluble drug entities: challenges and perspectives. *Eur J Pharm Sci* 45: 235-250.

Cheng KC, Li C, Uss AS (2008) Prediction of oral drug absorption in humans – from cultured cell lines and experimental animals. *Expert Opin Drug Metab Toxicol* 4: 581-590.

Christensen J, El-Gebali S, Natoli M, Sengstag T, Delorenzi M, Bentz S, Bouzourene H, Rumbo M, Felsani A, Siissalo S, Hirvonen J, Vila MR, Saletti P, Aguet M, Anderle P (2012) Defining new criteria for selection of cell-based intestinal models using publicly available databases. *BMC Genomics* 13: 274.

Coecke S, Blaauboer BJ, Elaut G, Freeman S, Freidig A, Gensmantel N, Hoet P, Kapoulas VM, Ladstetter B, Langley G, Leahy D, Mannens G, Meneguz A, Monshouwer M, Nemery B, Pelkonen O, Pfaller W, Prieto P, Proctor N, Rogiers V, Rostami-Hodjegan A, Sabbioni E, Steiling W, van de Sandt JJ. (2005) Toxicokinetics and metabolism. *Altern Lab Anim* 33 Suppl1: 147-175.

Crespi CL, Penman BW, Hu M (1996) Development of Caco-2 cells expressing high levels of cDNA-derived cytochrome P4503A4. *Pharm Res* 13: 1635-1641.

Crow JA, Borazjani A, Potter PM, Ross MK (2007) Hydrolysis of pyrethroids by human and rat tissues: examination of intestinal, liver and serum carboxylesterases. *Toxicol Appl Pharmacol* 221: 1-12.

Erhardt PW, Khupse R, Sarver JG and Trendel JA (2010) Prodrugs: Strategic Deployment, Metabolic Considerations, and Chemical Design Principles. *Burger's Medicinal Chemistry, Drug Discovery and Development*. 219–287.

Fogh J, Fogh JM, Orfeo T (1977) One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 59: 221-226.

Food and Drug Administration. FDA Guidance for industry, waiver of in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system. (Food and Drug Administration, Baltimore, MD, 2000). http://www.fda.gov/cder/guidance/3618fnl.htm (2012).

Gautrey H, Nicol F, Sneddon AA, Hall J, Hesketh J (2011) A T/C polymorphism is the GPX4 3'UTR affects the selenoprotein expression pattern and cell viability in transfected Caco-2 cells. *Biochim Biphys Acta* 1810: 584-591.

Geerts T, Vander Heyden T (2011) *In silico* predictions of ADME-Tox properties: drug absorption. *Comb Chem High Throughput Screen* 14: 339-361.

Hayeshi R, Hilgendorf C, Artursson P, Augustijns P, Brodin B, Dehertogh P, Fisher K, Fossati L, Hovenkamp E, Korjamo T, Masungi C, Maubon N, Mols R, Müllertz A, Mönkkönen J, O'Driscoll C, Oppers-Tiemissen HM, Ragnarsson EG, Rooseboom M, Ungell AL (2008) Comparison of drug transporter gene expression and functionality in Caco-2 cells from 10 different laboratories. *Eur J Pharm Sci* 35: 383-396.

Hidalgo IJ, Raub TJ, Borchardt RT (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal permeability. *Gastroenterology* 96: 736-749.

Hilgendrof C, Spahn-Langguth H, Rhedin M, Regårdh CG, Löwenadler B, Langguth P (2005) Selective downregulation of the MDR1 gene product in Caco-2 cells by stable transfection to prove its relevance in secretory drug transport. *Mol Pharm* 2: 64-73.

Hubatsch I, Ragnarsson EG, Artursson P (2007) Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat Protoc* 2: 2111-2119.

Imai T (2006) Human carboxylesterase isozymes: catalytic properties and rational drug design. *Drug Metab Pharmacokinet* 21: 173-185.

Imai T, Imoto M, Sakamoto H, Hashimoto M (2005) Identification of esterases expressed in Caco-2 cells and effects of their hydrolyzing activity in predicting human intestinal absorption. *Drug Metab Dispos* 33: 1185-1190.

Imai T, Ohura K (2010) The role of intestinal carboxylesterase in the oral absorption of prodrugs. *Curr Drug Metab* 11: 793-805.

Imai T, Taketani M, Shii M, Hosokawa M, Chiba K (2006) Substrate specificity of carboxylesterase isozymes and their contribution to hydrolase activity in human liver and small intestine. *Drug Metab Dispos* 34: 1734-1741.

Korjamo T, Honkakoski P, Toppinen MR, Niva S, Reinisalo M, Palmgrén JJ, Mönkkönen J (2005) Absorption properties and P-glycoprotein activity of modified Caco-2 cell lines. *Eur J Pharm Sci* 26: 266-279.

Korjamo T, Mönkkönen J, Uusitalo J, Turpeinen M, Pelkonen O, Honkakoski P (2006) Metabolic and efflux properties of Caco-2 cells stably transfected with nuclear receptors. *Pharm Res* 23: 1991-2001.

Kusano Y, Horie S, Morishita N, Shibata T, Uchida K (2012) Constitutive expression of an antioxidant enzyme, glutathione S-transferase P1, during differentiation of human intestinal Caco-2 cells. *Free Radic Biol Med* 53: 347-356.

Lamego J, Coroadinha AS, Simplício AL (2011) Detection and quantification of carboxylesterase 2 activity by capillary electrophoresis. *Anal Chem* 83: 881-887.

Lamego J, Cunha B, Peixoto C, Sousa MF, Alves PM, Simplício AL, Coroadinha AS (2012) Carboxylesterase 2 production and characterization in human cells: new insights into enzyme oligomerization and activity. *Appl Microbiol Biotechnol* doi: 10.1007/s00253-012-3994-3.

Lea MA, Ibeh C, Deutsch JK, Hamid I, desBordes C (2010) Inhibition of growth and induction of alkaline phosphatase in colon cancer cells by flavonols and flavonol glycosides. *Anticancer Res* 30: 3629-3635.

Li J, Volpe DA, Wang Y, Zhang W, Bode C, Owen A, Hidalgo IJ (2011) Use of transporter knockdown Caco-2 cells to investigate the in vitro efflux of statin drugs. *Drug Metab Dispos* 39: 1196-1202.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25: 402-408.

Malpique R, Katsen-Globa A, Carrondo MJ, Zimmermann H, Alves PM (2007) Cryopreservation in micro-volumes: impact upon Caco-2 colon adenocarcinoma cell proliferation and differentiation. *Biotechnol Bioeng* 98: 155-166.

Mutskov V, Felsenfeld G (2004) Silencing of transgene transcription precedes methylation of promoter DNA and histone H3 lysine 9. *EMBO J* 23: 138-149.

Natoli M, Leoni BD, D'Agnano I, D'Onofrio M, Brandi R, Arisi I, Zucco F, Felsani A (2011) Cell growing density affects the structural and functional properties of Caco-2 differentiated monolayer. *J Cell Physiol* 226: 1531-1543.

Natoli M, Leoni BD, D'Agnano I, Zucco F, Felsani A (2012) Good Caco-2 cell culture practices. *Toxicol In Vitro* doi: 10.1016/j.tiv.2012.03.009.

Ohura K, Sakamoto H, Ninomiya S, Imai T (2010) Development of a novel system for estimating human intestinal absorption using Caco-2 cells in the absence of esterase activity. *Drug Metab Dispos* 38: 323-331.

O' Neill MJ, Guo J, Byrne C, Darcy R, O' Driscoll CM (2011) Mechanistic studies on the uptake and intracellular trafficking of novel cyclodextrin transfection complexes by intestinal epithelial cells. *Int J Pharm* 413: 174-183.

Pang S, Urquhart P, Hooper NM (2004) N-glycans, not the GPI anchor, mediate the apical targeting of a naturally glycosylated, GPI-anchored protein in polarized epithelial cells. *J Cell Sci* 117: 5079-5086.

Pindel EV, Kedishvili NY, Abraham TL, Brzezinski MR, Zhang J, Dean RA, Bosron WF (1997) Purification and cloning of a broad substrate specificity human liver carboxylesterase that catalyzes the hydrolysis of cocaine and heroin. *J Biol Chem* 272: 14769–14775.

Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simon-Assmann P, Haffen K, Fogh J, Zweibaum A (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* 47: 323-330.

Press B, Di Grandi D (2008) Permeability for intestinal absorption: Caco-2 assay and related issues. *Curr Drug Metab* 9: 893-900.

Prieto P, Hoffmann S, Tirelli V, Tancredi F, González I, Bermejo M, De Angelis I (2010) An exploratory study of two Caco-2 cell models for oral absorption: a report on their within-laboratory and between-laboratory variability, and their predictive capacity. *Altern Lab Anim* 38: 367-386.

Reis JM, Sinkó B, Serra CH (2010) Parallel artificial membrane permeability assay (PAMPA) – Is it better than Caco-2 for human passive permeability prediction? *Mini Rev Med Chem* 10: 1071-1076.

Rodolosse A,Barbat A, Chantret I, Lacasa M, Brot-Laroche E, Zweibaum A, Rousset M (1998) Selecting agent hygromycin B alters expression of glucose-regulated genes in transfected Caco-2 cells. *Am J Physiol* 274: G931-G938.

Ross MK, Crow JA (2007) Human carboxylesterases and their role in xenobiotic and endobiotic metabolism. *J Biochem Mol Toxicol* 21: 187-196.

Roth WJ, Lindley DJ, Carl SM, Knipp GT (2012) The effects of intralaboratory modifications to media composition and cell source on the expression of pharmaceutically relevant transporters and metabolizing genes in the Caco-2 cell line. *J Pharm Sci* 101: 3962-3978

Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F (2005) The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol* 21: 1-26.

Sanghani SP, Quinney SK, Fredenburg TB, Sun Z, Davis WI, Murry DJ, Cummings OW, Seitz DE, Bosron WF (2003) Carboxylesterases expressed in human colon tumor tissue and their role in CPT-11 hydrolysis. *Clin Cancer Res* 9: 4983-4991.

Schmiedlin-Ren P, Thummel KE, Fisher JM, Paine MF, Watkins PB (2001) Induction of CYP3A4 by 1 alpha,25-dihydroxyvitamin D3 is human cell line-specific and is unlikely to involve pregnane X receptor. Drug Metab Dispos 29: 1446-1453.

Shah P, Jogani V, Bagchi T, Misra A (2006) Role of Caco-2 cell monolayers in prediction of intestinal drug absorption. *Biotechnol Prog* 22: 186-198.

Siissalo S, Laitinen L, Koljonen M, Vellonen KS, Kortejärvi H, Urtti A, Hirvonen J, Kaukonen AM (2007) Effect of cell differentiation and passage number on the expression of efflux proteins in wild type and vinblastine-induced Caco-2 cell lines. *Eur J Pharm Biopharm* 67: 548-554.

Simon-Assman P, Turck N, Sidhoum-Jenny M, Gradwohl G, Kedinger M (2007) *In vitro* models of intestinal epithelial cell differentiation. *Cell Biol Toxicol* 23: 241-256.

Ungell AL (2004) Caco-2 replace or refine? *Drug Discov Today Technol* 1: 423-430.

Volpe DA (2008) Variability in Caco-2 and MDCK cell-based intestinal permeability assays. *J Pharm Sci* 97: 712-725.

Volpe DA (2010) Application of method suitability for drug permeability classification. *AAPS J* 12: 670-678.Volpe DA, Faustino PJ, Ciavarella AB, Asafu-Adjaye EB, Ellison CD, Yu LX, Hussain AS (2007) Classification of drug permeability with a Caco-2 cell monolayer assay. *Clinical Research and Regulatory Affairs* 24: 39-47.

Wierdl M, Tsurkan L, Hyatt JL, Edwards CC, Hatfield MJ, Morton CL, Houghton PJ, Danks MK, Redinbo MR, Potter PM (2008) An improved human carboxylesterase for enzyme/prodrug therapy with CPT-11. *Cancer Gene Ther* 15: 183-192.

Williams ET, Bacon JA, Bender DM, Lowinger JJ, Guo WK, Ehsani ME, Wang X, Wang H, Qian YW, Ruterbories KJ, Wrighton SA, Perkins EJ (2011) Characterization of the expression and activity of carboxylesterases 1 and 2 from the beagle dog, cynomolgus monkey, and human. *Drug Metab Dispos* 39: 2305-2313.

Xu G, Zhang W, Ma MK, McLeod HL (2002) Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of Irinotecan. *Clin Cancer Res* 8: 2605-2611.

SECTION 5

Discussion and Future Work

Contents

The main goal of the work presented in this thesis was to increase the fundamental knowledge on human Carboxylesterase 2 (hCES2) and to improve Caco-2 cells by increasing the levels of this enzyme, crucial in the metabolism of ester containing drugs and prodrugs. The main contributions to that goal are highlighted in this section with a special focus on the future envisioned developments.

5.1 Setting new analytical approaches

Currently available tools enabling the assessment of carboxylesterase (CES) expression and activities have several limitations, as detailed in Section 2, such as the failure to associate protein expression with activity quantification as well as the inability to distinguish different CESs and quantify their specific activities. For the proper study of CES2 activity in complex biological samples, proper tools had to be developed and were described in Section 2. Taking advantage of BNPP, a general and irreversible CES inhibitor, and loperamide, a selective CES2 inhibitor, CESs were evaluated through capillary electrophoresis for the first time, using a general CES substrate, 4-MUBA. First, commercially available enzymes were used, with the method later extended to the evaluation of increasingly complex biological samples such as human cell extracts and mammalian sera. A workflow where an unknown sample may be sequentially tested for its hydrolytic activity towards a general CES substrate, such as 4-MUBA, in the absence and presence of these inhibitors was proposed. This method, using up to 7-fold less sample than the classical spectrophotometric methods, showed to be appropriate when several components of the reaction mixture absorb at the same wavelength.

The future applications of the developed methodology are broad, ranging from the possibility of testing different samples to the evaluation of other CESs, or even other esterases since the appropriate inhibitors or substrates are used. It may also be used with other separation techniques like HPLC.

The development of new approaches for the differentiation and characterisation of CES activity is of great relevance in this field where much attention is being dedicated to the comparison of the specific activities found in different mammals, namely those used in preclinical studies by pharmaceutical companies (Williams et al. 2011; Bahar et al. 2012). Foreseen is the future development of new analytical tools enabling differentiation and characterisation of CES activity in whole-living cells, where the enzymes are retained in a preserved and more physiological cellular environment. In fact specific fluorescent probes have already been developed and characterised for their specificity for different CESs (Wang et al. 2011). The application of these probes for live imaging was shown to be possible (Hakamata et al. 2011) raising the possibility of further applications that may help to understand the differences between enzyme activity and protein expression previously reported (Ross et al. 2012).

5.2 Further understanding hCES2

Having developed the tools allowing its proper study in biological samples, human carboxylesterase 2 (hCES2) production was for the first time performed and described, in Section 3, in suspension adapted HEK-293T cells. The strategy used allowed no need for additional of N-terminal signal sequences or the modification of the ER retention sequence to promote hCES2 secretion with the addition of an in frame C-terminally localised 10x histidine tag. This, in turn, allowed the purification of the protein through affinity chromatography. Although a very satisfactory purity level was obtained, the achieved yield was in line with a recent report (Hatfield et al. 2010), leaving space for future improvements in the purification process, which the research group is already pursuing.

The manufacturing of hCES2 enabled further comprehension of its behaviour: it may present itself as active and inactive oligomers when it was previously reported to be exclusively found as 60 kDa monomers (Pindel et al. 1997). We have

hypothesised that hCES2 monomers may associate forming structures of higher complexity, depending on the environment where the protein is. The further characterisation of the observed oligomers may be performed, in the future, through size exclusion chromatography; nonetheless, improvement in the purification process is needed to increase yields, allowing these types of studies. Whether these oligomers exist in the human organism and what is their relevance, are curious still unanswered questions.

The future applications of this produced human recombinant CES2 are massive. Due to the high purity levels achieved it will be used in crystallographic studies. As, so far, the only human CES structure known is that of carboxylesterase 1 (hCES1; Bencharit et al. 2003) the determination of hCES2 structure will be an important contribution to this field. It may also be used in studies aiming at the discovery of new hCES2 inhibitors or in the investigation of the kinetics towards newly developed ester containing drugs or prodrugs.

The novel properties of hCES2 challenge the current knowledge about CESs. Several puzzling questions arise concerning the general properties of these proteins as well as their evolutionary path.

May oligomerization be a shared property of CESs? As mentioned before, hCES2 ability to form organised structures and being active in forms other than monomers may not be an unusual property and suggest a broader CES-shared property. For instance, it was previously reported that esterase D can form active dimers of 35 kDa monomers (Okada and Wakabayashi 1988) and hCES1 is active as trimers, monomers, and dimers with the hexamers being inactive (Takai et al. 1997; Bencharit et al. 2003; Crow et al. 2007). In fact, homology modelling prediction of hCES2 structure based on hCES1 has highlighted the presence of the Z-site region, which modulates hCES1 trimer–hexamer equilibrium, in the C-terminus of hCES2 (Vistoli et al. 2010). Further studies concerning CES oligomerization are thus

envisioned, being important to carefully design and conduct the studies so that the most relevant data, from the biological point of view, are obtained.

What is the importance of glycosylation in protein stability/solubilisation and activity? As previously mentioned, native hCES2 is known to be sensitive to Endo H digestion (Pindel et al. 1997). Purified and active human recombinant CES2 bears complex-type glycosylation and not high-mannose type, thus sensitive to PNGase F, a not so surprising characteristic since further glycosylation modifications are expected by the Golgi apparatus on secreted proteins. This glycosylation pattern has previously been shown in rat carboxylesterases (Yan et al. 1995): it was observed that both liver microsomal and secreted forms existed, with the intracellular form sensitive to Endo H digestion and the secreted form sensitive to PNGase F. Rat hydrolase S was shown to be secreted from rat primary culture of hepatocytes, unlike rat hydrolases A and B which are intracellular. The first does not contain the HXEL C-terminus consensus sequence, unlike the non-secreted forms, having also more glycosylation sites (Yan et al. 1994; Yan et al. 1995). Moreover, the native deglycosylation of hydrolases A and B, purified from rat liver microsomes, has shown no effect on enzyme activity, although an increased adsorption to glass and plastic was observed for carbohydrate-free hydrolase A (Morgan et al. 1994). As previously hypothesised (Morgan et al. 1994) these data may point towards a higher relevance of glycosylation for protein stability and solubilisation and not as much for enzymatic activity of mature proteins. Other investigators have also highlighted the importance to look at the glycosylation sites in order to acquire a deeper understanding of the catalytic mechanism of CESs (Satoh and Hosokawa 2006).

Interesting insights were also previously reported from recombinant expression of different CESs. Hydrolase C, a rat carboxylesterase very similar to rat hydrolase B, was shown to be inactive when expressed in *Escherichia coli*, a prokaryotic organism, but active when expressed in the baculovirus/insect cell

(*Sporodeptra fugiperda* 21 – Sf21) system (Yan et al. 1995b). Curiously, it was reported that for full recombinant hCES1 activity, expressed in baculovirus-insect cell system, proper glycosylation (especially N-linked glycosylation) was needed (Kroetz et al. 1993). These combined data may point towards differences in the glycosylation function not only between intra and extracellular forms but also across different CESs. The reasoning behind these differences remains unknown.

To understand if the post-translational modifications encountered in mature human secreted CES2 have an impact on protein stability and solubility, an experimental approach could be envisioned where the sugar moieties would be removed under native conditions and these data would confirm the previous findings in rat liver carboxylesterases (Morgan et al. 1994). Moreover, to address the impact of post-translational modifications on the maturation of hCES2, site-directed mutagenesis should help to understand if glycosylation is needed for the proper folding and activity of this enzyme. A similar approach may be used to study the role and the impact of the cysteine residues involved in the formation of disulfide bonds.

What came first: intra or extracellular forms of CESs in mammals? As previously reported, humans do not have CESs in the plasma (Li et al. 2005). Nonetheless, other mammals, such as rats and mice, have several intra and extracellular carboxylesterase forms (Holmes et al. 2010). Is carboxylesterase secretion a common ancestral property in mammals that humans lost throughout evolution or is it an acquired characteristic that humans did not need to have? Data from rat, shown that native hydrolase S was exclusively secreted from the liver, as no mRNA was found in other tissues, and differed from the liver microsomal carboxylesterase form only in the glycosylation pattern. These previously reported results, may point towards the primary existence of carboxylesterases in the intracellular form. Supporting this evidence, the intracellular levels of hydrolase S were shown to be lower than the ones of hydrolase A and B. Moreover, both forms of

hydrolase S, intra and extracellular, were shown to be regulated by inducer and suppressor xenobiotics, at the protein and mRNA levels (Yan et al. 1995). Nonetheless, these data do not explain why hydrolase S lost the HXEL C-terminus motif or why humans do not have CES secretion.

A non-human organism where human cells were allowed to grow and develop would allow gaining new insights towards the possible influence of the environment of the organism for the development of the human cells as well as on CES regulation and activity. A model organism was already created (Strom et al. 2010) and patented: the FRG™ mouse. This commercially available (Yecuris; Portland, U.S.A.) chimeric triple knockout (KO) model for fumarylacetoacetate hydrolase, recombinant activating gene, and interleukin-2 receptor subunit gamma (*Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$), allows human hepatocytes to grow inside the liver of the animal and almost entirely replace native hepatocytes. *Rag2* and *Il2rg* absence renders these mice immunodeficient, allowing the prompt acceptance of engrafted human hepatocytes. *Fah* KO leads to chronic liver damage in the mice, which is prevented by 2-(2-nitro-4-trifluoro-methylbenzoyl)1,3-cyclohexedione (NTBC), keeping them healthy. Upon human hepatocyte transplantation, the removal of NTBC application provides the selective advantage for the repopulation of the injured mouse liver (Strom et al. 2010). This model provides an opportunity for evaluating the expression and activity of hCES2, as well as other CESs, in human hepatocytes grown in a non-human organism in order to understand if changes in the enzymatic profile arise not only in comparison to the parental transplanted population but also throughout the repopulation stages. This strategy was already applied in the study of other drug metabolising enzymes, such as the cytochrome P450 (CYP) family, in various chimeric models (Strom et al. 2010). In the *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ model, it was shown that the expression levels of different CYP genes as well as their transcriptional inducers, pregnane X receptor (PXR) and constitutive androstane receptor (CAR; Lim and Huang 2008), were similar to the ones found in the donor population (Azuma et al. 2007). To

our best knowledge, no data concerning CES expression was reported using this strategy.

This approach would also allow comparing the sera from healthy (control), KO, and humanised mice at different stages of repopulation, as mentioned. Through CES protein and activity specific detection in the sera, it would be possible to investigate whether a decrease in mice CES expression or activity levels occurred throughout time. If so, it would point towards a decrease in CES secretion due to the decrease in the amount of mice cells in the liver, as would be expected. If not, it might mean that mice cells were able to sense the eventual decrease of CES levels in the blood and increase secretion of more active enzymes. Another possibility would be that the repopulating cells might somehow acquire (or re-acquire) the ability to secrete CESs or that they can reprogram themselves or be reprogramed by the environment to do so. This would be a tremendous result, providing new insights towards mice and human CESs expression and activity regulation, strengthening the quest of understanding why human cells do not secrete CESs while other animals do.

5.3 Improving Caco-2 cells

Fitted with a deep knowledge about hCES2, Caco-2 cells were successfully genetically engineered, obtaining a population with increased *hCES2* mRNA, protein expression and activity levels, as described in Section 4, without changing crucial Caco-2 characteristics, such as differentiation and polarisation. Through clonal selection by limiting dilution, it was possible to generate a Caco-2 CES2 cell line that may be suitable in the future for the coupling of *in vitro* intestinal transport and metabolism evaluation of pharmaceutically relevant compounds. It was previously highlighted that the coupling of these two mechanisms would render an ideal *in vitro* permeability model (Balimane and Chong 2005).

Through stability evaluation of hCES2 expression throughout passaging and differentiation, an obvious decay was observed rendering this newly developed cell

line unsuitable for drug testing at higher passages. Testing the permeability and metabolism of ester containing drugs and prodrugs will be possible through the establishment of a large cell bank at a low passaging level, as previously reported (Crespi et al. 1996) or, alternatively, through the transient transfection of Caco-2 with the *hCES2* gene.

The reasons for the observed expression instability remain unclear. The general problem to stably modify Caco-2 cells was mentioned before (Korjamo et al. 2006). It is apparently not related to the chosen methodology for gene expression as the same instability was previously reported following a different strategy in terms of the chosen transgene, cell transfection and selection pressure (Crespi et al. 1996). Our data also point towards an independency of selection pressure used since the decline in hCES2 activity was detected both in the presence and in the absence of the antibiotic.

As there are several reports for the stable transfection of Caco-2 cells, with different transgenes, without effectively showing cell line stability at later stages, it may be envisioned that a possible common mechanism exists through which the cells effectively repress the expression of exogenous genes (Crespi et al. 1996; Hilgendorf et al. 2004; Gautrey et al. 2011). In the case of hCES2, our data shows that the regulation is not at the activity level since it is accompanied by a decrease in protein expression. As mentioned in Section 4, due to a clear response of *hCES2* mRNA during the differentiation period in Caco-2 CES2 stably transfected population, we hypothesise that transcription may be at the level of regulation of exogenous hCES2 expression. We cannot exclude that repression mechanisms may exist at the DNA level.

To understand the level where regulation of the expression occurs, several approaches may be followed in the future. If *hCES2* mRNA also reflects the verified instability, different efforts may be envisioned to understand if this is due to decay in the transcription or the turnover rate of mRNA. Xenobiotic inducers of CES

expression, known to act at the transcription level, may help to differentiate between these two hypotheses. The usage of these inducers, as a solution towards a more stable cell line suitable for drug testing at higher passaging levels, seems unreasonable since they would affect the expression of other key enzymes and other proteins. Another possibility is the usage of CES specific substrates as a way to induce or keep the expression of these enzymes. Similar strategies may be followed to further investigate whether epigenetic mechanisms are silencing the transgene at the DNA level. If such mechanisms are proven to be regulating the expression of the transgene, different approaches towards its stable expression must be pursued. One of these may be the targeted integration of CES2 following cassette exchange on a previously screened stable chromosomal locus, an approach successfully followed in the past in other human cell lines (Schucht et al. 2006; Coroadinha et al. 2006). The use of insulators sequences may also be an option if the problem arises from transcriptional silencing. Chromatin insulators are regulatory elements known to reduce the interaction between integrated expression vectors and the surrounding cell genome. These interactions may be a source of expression instability associated with the integrated transgenes (Emery 2011; van Bortle and Corces 2012).

In a time where there are ethical, economic, and regulatory needs for better alternatives to animal experimentation, this approach has the potential of becoming an invaluable tool. Further metabolic improvements may be envisioned, such as *hCES1* down-regulation and *CYP3A4* up-regulation, leading to the establishment of an increasingly better *in vitro* intestinal permeability tool that may be used for research purposes as well as pre-clinical studies contributing to the 3R policy, to Replace, Reduce, and/or Refine laboratory animal use when possible (Schiffelers et al. 2012; Wells 2011).

5.4 Final remarks

Overall, the work described in this thesis contributed significantly to the progress of the current state of the art on xenobiotic metabolism and permeability fields. Important advancements were made which contributed to the improvement of tools for human carboxylesterases studies: a new capillary electrophoresis method enabling the quantification of specific hCES2 activity, using up to 7 times less sample than classical spectrophotometric methods, was developed. This methodology, having the potential to be extended for the quantification of other hCESs, allows performing determinations even when substrate, product, or inhibitors absorb at the same wavelength.

A significant contribution was also made to the current knowledge on carboxylesterases, through the production and purification of hCES2 in human cells, showing, for the first time, the ability of this enzyme to oligomerize. Moreover, through the secretion of the enzyme, not only were the production yields boosted, achieving 50 $mg.L^{-1}$, but also allowed to find the addition of a C-terminal Histidine tag was enough to potentiate hCES2 secretion. In a time when increased awareness to interspecies variation exists, as well as growing attention is being devoted to these enzymes in anti-cancer combined therapies, the contributions of better human carboxylesterases manufacturing techniques and increased understanding on how these enzymes behave are invaluable.

Additionally, a modified intestinal permeability model, based on the widely used Caco-2 cell line, was generated. A population of Caco-2 with two-fold increased hCES2 activity was obtained and through this, a cell clone was isolated with a six-fold increase in the activity of the protein. The establishment of this cell line constitutes an important contribution to the current *in vitro* tools used to determine the intestinal permeability, in an urgent time for the effective application of the 3R policy.

5.5 Acknowledgements

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5.6 References

Azuma H, Paulk N, Ranade A, Dorrell C, Al-Dhalimy M, Ellis E, Strom S, Kay MA, Finegold M, Grompe M (2007) Robust expansion of human hepatocytes in *Fah-/-/Rag2-/-/Il2rg-/-* mice. *Nat Biotechnol* 25: 903-910.

Bahar FG, Ohura K, Ogihara T, Imai T (2012) Species difference of esterase expression and hydrolase activity in plasma. *J Pharm Sci* (in press) doi: 10.1002/jps.23258.

Balimane PV, Chong S (2005) Cell culture-based models for intestinal permeability: a critique. *Drug Discov Today* 10: 335-343.

Bencharit S, Morton CL, Xue Y, Potter PM, Redinbo MR (2003) Structural basis of heroin and cocaine metabolism by a promiscuous human drug-processing enzyme. *Nat Struct Biol* 10: 349–356.

Coroadinha AS, Schucht R, Gama-Norton L, Wirth D, Hauser H, Carrondo MJ (2006) The use of recombinase mediated cassette exchange in retroviral vector producer cell lines: predictability and efficiency by transgene exchange. *J Biotechnol* 124: 457-468.

Crespi CL, Penman BW, Hu M (1996) Development of Caco-2 cells expressing high levels of cDNA-derived cytochrome P4503A4. *Pharm Res* 13: 1635-1641.

Crow JA, Borazjani A, Potter PM, Ross MK (2007) Hydrolysis of pyrethroids by human and rat tissues: examination of intestinal, liver and serum carboxylesterases. *Toxicol Appl Pharmacol* 221: 1–12.

Emery DW (2011) The use of chromatin insulators to improve the expression and safety of integrating gene transfer vectors. *Hum Gene Ther* 22: 761-774.

Gautrey H, Nicol F, Sneddon AA, Hall J, Hesketh J (2011) A T/C polymorphism is the GPX4 3'UTR affects the selenoprotein expression pattern and cell viability in transfected Caco-2 cells. *Biochim Biphys Acta* 1810: 584-591.

Hakamata W, Machida A, Oku T, Nishio T (2011) Design and synthesis of an ER-specific fluorescent probe based on carboxylesterase activity with quinone methide cleavage process. *Bioorg Med Chem Lett* 21: 3206-3209.

Hatfield MJ, Tsurkan L, Hyatt JL, Yu X, Edwards CC, Hicks LD, Wadkins RM, Potter PM (2010) Biochemical and molecular analysis of carboxylesterase-mediated hydrolysis of cocaine and heroin. *Br J Pharmacol* 160: 1916–1928.

Hilgendorf C, Spahn-Langguth H, Rhedin M, Regårdh CG, Löwenadler B, Langguth P (2005) Selective downregulation of the MDR1 gene product in Caco-2 cells by stable transfection to prove its relevance in secretory drug transport. *Mol Pharm* 2: 64-73.

Holmes RS, Wright MW, Laulederkind SJ, Cox LA, Hosokawa M, Imai T, Ishibashi S, Lehner R, Miyazaki M, Perkins EJ, Potter PM, Redinbo MR, Robert J, Satoh T, Yamashita T, Yan B, Yokoi T, Zechner R, Maltais LJ (2010) Recommended nomenclature for five mammalian carboxylesterase gene families: human, mouse and rate genes and proteins. *Mamm Genome* 21: 427–441.

Kroetz DL, McBride OW, Gonzalez FJ (1993) Glycosylation-dependent activity of baculovirus-expressed human liver carboxylesterases: cDNA cloning ad characterization of two highly similar enzyme forms. *Biochemistry* 32: 11606-11617.

Lim YP, Huang JD (2008) Interplay of pregnane X receptor with other nuclear receptors on gene regulation. *Drug Metab Pharmacokinet* 23: 14-21.

Morgan EW, Yan B, Greenway D, Petersen DR, Parkinson A (1994) Purification and characterization of two rat liver microsomal carboxylesterases (hydrolase A and B). *Arch Biochem Biophys* 315: 495-512.

Okada Y, Wakabayashi K (1988) Purification and characterization of esterases D-1 and D-2 from human enterocytes. *Arch Biochem Biophys* 263: 130–136.

Pindel EV, Kedishvili NY, Abraham TL, Brzezinski MR, Zhang J, Dean RA, Bosron WF (1997) Purification and cloning of a broad substrate specificity human liver carboxylesterase that catalyses the hydrolysis of cocaine and heroin. *J Biol Chem* 272: 14769–14775.

Ross MK, Borazjani A, Wang R, Crow JA, Xie S (2012) Examination of the carboylesterase phenotype in human liver. *Arch Biochem Biophys* 522: 44-56.

Satoh T, Hosokawa M (2006) Structure, function and regulation of carboxylesterases. *Chem Biol Interact* 162: 195–211.

Schiffelers MJ, Blaauboer BJ, Hendriksen CF, Bakker WE (2012) Regulatory acceptance and use of 3R models: a multilevel perspective. *ALTEX* 29:287-300.

Schucht R, Coroadinha AS, Zanta-Boussif MA, Verhoeyen E, Carrondo MJ, Hauser H, Wirth D (2006) A new generation of retroviral producer cells: predictable and stable virus production by Flp-mediated site-specific integration of retroviral vectors. *Mol Ther* 14: 285-292.

Strom SC, Davila J, Grompe M (2010) Chimeric mice with humanized liver: tools for the study of drug metabolism, excretion, and toxicity. *Methods Mol Biol* 640: 491-509.

Takai S, Matsuda A, Usami Y, Adachi T, Sugiyama T, Katagiri Y, Takematsu M, Hirano K (1997) Hydrolytic profile for ester- or amide-linkage by carboxylesterases pI 5.3 and 4.5 from human liver. *Biol Pharm Bull* 20: 869–873.

Van Bortle K, Corces VG (2012) Nuclear organization and genome function. *Annu Rev Cell Dev Biol* doi: 10.1146/annurev-cellbio-101011-155824.

Vistoli G, Pedretti A, Mazzolari A, Testa B (2010) Homology modelling and metabolism prediction of human carboxylesterase-2 using docking analysis by GriDock: a parallelized tool based on Auto-Dock 4.0. *J Comput Aided Mol Des* 24: 771–787.

Wang J, Williams ET, Bourgea J, Wong YN, Patten CJ (2011) Characterization of recombinant human carboxylesterases: fluorescein diacetate as a probe substrate for human carboxylesterase 2. *Drug Metab Dispos* 39: 1329-1333.

Wells DJ (2011) Animal welfare and the 3Rs in European biomedical research. *Ann N Y Acad Sci* 1245: 14-16.

Williams ET, Bacon JA, Bender DM, Lowinger JJ, Guo WK, Eshani ME, Wang X, Wang H, Qian YW, Ruterbories KJ, Wrighton SA, Perkins EJ (2011) Characterization of the expression and activity of carboxylesterases 1 and 2 from the beagle dog, cynomolgus monkey, and human. *Drug Metab Dispos* 39: 2305–2313.

Yan B, Yang D, Brady M, Parkinson A (1994) Rat kidney carboxylesterase. *J Biol Chem* 269: 29688-29696.

Yan B, Yang D, Bullock P, Parkinson A (1995) Rat serum carboxylesterase. Cloning, expression, regulation, and evidence of secretion from liver. *J Biol Chem* 270: 19128–19134.

Yan B, Yang D, Parkinson A (1995b) Cloning and expression of Hydrolase C, a member of the rat carboxylesterase family. *Arch Biochem Biophys* 317: 222-234.

APPENDICES

Contents

A.1 CES2 protein across different species

The following table is the extended version of Table 1.2, presented in Section 1, containing all the hits until hCES1 sequence (one hundred and sixty six hits in total).

As mentioned in Section 1, a BLAST-P search was performed in ESTHER database against all protein sequences available (http://bioweb.ensam.inra.fr/ESTHER/general?what=index), using as query, the amino acid (a.a.) sequence of hCES2. All the hits were considered, except those classified by the database as belonging to the Carb_B_FragtPseudo group, as these constitute incomplete sequences. Moreover, the hits were cross-checked against UniProtKB/Swiss-Prot database (http://www.uniprot.org) and only those having an identifiable entry were considered (forty eight in total, for Table 1.2; one hundred and fifty two for Table A.1).

The default parameters of the program, in the mentioned webpage, were used: substitution matrix – blocks of amino acid substitution matrix 62 (BLOSUM62); gapped alignment; gap penalties: existence – 11; extension – 1.

As also mentioned in Section 1, the sequence identities towards the used query (ID), as well as additional information are provided. These include: protein accession numbers in ESTHER and UniProt databases; alternative protein entries (italicized); protein name and function information available at UniProt database; gene designation (italicized) when existing; GeneBank accession number; level of experimental evidence of protein existence (evp); length of the full protein (#AA) as well as the last four amino acids. Moreover, the score (S; in bits) for each alignment as well as the expectation value (E-value) are shown. Additional references, not mentioned in Section 1, may be found in the end of this section.

The UniProt evp levels were followed (http://www.uniprot.org/manual/protein_existence). The criteria for the five types of evidence for the existence of a protein are herein transcribed:

Evidence at protein level - experimental evidence for the existence of the protein. Criteria: partial or complete Edman sequencing, identification by mass spectrometry, X-ray or NMR structure, good quality protein-protein interaction or detection of the protein by antibodies.

Evidence at transcript level - existence of a protein has not been strictly proven; expression data (such as existence of cDNA(s), RT-PCR or Northern blots) indicate the existence of a transcript.

Inferred by homology - existence of a protein is probable because clear orthologs exist in closely related species.

Predicted - entries without evidence at protein, transcript, or homology levels.

Uncertain - existence of the protein is unsure.

Table A.1 CES2 protein across different species

Organism / UniProt name	UNIPROT	ID %	# AA	Last 4 AA	ESTHER	GeneBank	S / E-value	Function
***Homo sapiens* (Human – class *Mammalia*)**								
Carboxylesterase 2 (Cocaine esterase)	O00748 (*A8K367*, *Q4G0E9*)	100	559	HTEL	human-2cxes	Y09616.1	1136 / 0.0	Detoxification of xenobiotics and activation of ester and amide prodrugs. High catalytic efficiency for hydrolysis of cocaine, 4-MUBA, heroin and 6-monoacetylmorphine. (Pindel et al. 1997)
Carboxylesterase 3	Q6UWW8	46	571	QEDL	human-est3	XM_016735, AK097538, BC053670, AY358609, EU595874, CH471092, AC009084	483 / 1e-136	Carboxylesterase activity (evp: protein level; Sanghani et al. 2004)
Liver carboxylesterase 1	P23141	46	567	HIEL	human-cxest	AB119998, AB119996, M65261	473 / 1e-133	Carboxylesterase activity (evp: protein level)
***Pan troglodytes* (Chimpanzee – class *Mammalia*)**								
Uncharacterised Protein	H2QBA6	97	623	HTEL	pantr-h2qba6	AACZ03102754.1	1111 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised Protein	H2RA50	47	571	QEDL	pantr-h2ra50	AACZ03102749, AACZ03102750, AACZ03102751	478 / 1e-135	Hydrolase activity (evp: predicted)
Uncharacterised Protein	H2R2Y7	46	567	HIAL	pantr-h2r2y7	AACZ03103390	483 / 1e-136	Hydrolase activity (evp: predicted)
***Gorilla gorilla* (Lowland gorilla - class *Mammalia*)**								
Uncharacterised Protein	G3RPG8 (*G3QYW6, G3QYX2*)	95	623	HTEL	gorgo-g3qyw6	-	1087 / 0.0	Hydrolase activity (evp: predicted)

***Pongo abelii* (Sumatran orangutan - class *Mammalia*)**								
Uncharacterised Protein	H2NR54	93	623	HTEL	ponab-h2nr54	-	1071 / 0.0	Hydrolase activity (evp: predicted)
Carboxylesterase 3	Q5RCL7	49	569	QEDL	ponab-est3	CR857194, CR858253	513 / 1e-146	Carboxylesterase activity (evp: transcript level)
Putative uncharacterised protein	Q5R545	46	566	HIEL	ponpy-q5r545	CR861029	473 / 1e-134	Hydrolase activity (evp: transcript level)
***Nomascus leucogenys* (Northern white-cheeked gibbon - class *Mammalia*)**								
Uncharacterised Protein	G1QVW2	91	606	HTEL	nomle-g1qvw2	ADFV01013581.1	1031 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised protein (*CES3*)	G1QVY9	47	571	QEDL	nomle-g1qvy9	ADFV01013581	482 / 1e-136	Hydrolase activity (evp: predicted)
Uncharacterised protein (*CES1*)	G1QM39	45	567	HIEL	nomle-g1qm39	ADFV01012901, ADFV01012902, ADFV01012903, ADFV01012904, ADFV01012905, ADFV01012906, ADFV01012907	479 / 1e-135	Hydrolase activity (evp: predicted)
***Papio hamadryas* (Hamadryas baboon - class *Mammalia*)**								
Ces2	B5TZ26	90	561	HTEL	papha-b5tz26	FJ147179.1	1030 / 0.0	Hydrolase activity (evp: transcript level; Holmes et al. 2009)
Carboxylesterase 1	B5TZ25	46	567	HIEL	papha-b5tz25	FJ147178	475 / 1e-134	Hydrolase activity (evp: transcript level; Holmes et al. 2009)
***Macaca mulatta* (Rhesus monkey - class *Mammalia*)**								
Uncharacterised Protein	F6UNJ2	88	543	HTEL	macmu-f6unj2	-	1004 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised Protein	G7NQ39	88	543	HTEL	macmu-g7nq39	CM001272.1	1003 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised Protein	F7AA58	48	534	HIEL	macmu-f7aa58	-	484 / 1e-137	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
Uncharacterised protein (*CES5A*)	F7AIM4	47	581	VIFL	macmu-f7aim4	-	478 / 1e-135	Hydrolase activity (evp: predicted)
Uncharacterised Protein	F7AI42 (*F7AI34*)	46	566	HIEL	macmu-f7ai42	-	478 / 1e-135	Hydrolase activity (evp: predicted)

***Callithrix jacchus* (White-tufted-ear marmoset - class *Mammalia*)**								
Uncharacterised Protein	F6ZPL6 (*F6Z7R7*)	83	620	HTEL	calja-f6zpl6	ACFV01013743.1, ACFV01013744.1, ACFV01013745.1	946 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised Protein	F6Y4M2, (*F6Y501*)	58	530	QEEL	calja-f6y4m2	ACFV01013743, ACFV01013744, ACFV01013745, ACFV01013746, ACFV01013747, ACFV01013748, ACFV01013749	618 / 1e-177	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
***Ailuropoda melanoleuca* (Giant panda - class *Mammalia*)**								
Putative uncharacterised protein	D2H9C9 (*G1MFN8*)	77	534	HTEL	ailme-d2h9c9	GL192596.1, ACTA01043044.1	852 / 0.0	Hydrolase activity (evp: predicted; Li et al. 2010)
Putative uncharacterised protein (*CES5A*)	D2GTV3 (*G1MDU0*)	49	551	SSAP	ailme-d2gtv3	GL192338, ACTA01072786	493 / 1e-139	Hydrolase activity (evp: predicted; Li et al. 2010)
Uncharacterised protein (*CES1*)	G1MDR5	46	565	HVEL	ailme-g1mdr5	ACTA01064786, ACTA01072786	482 / 1e-136	Hydrolase activity (evp: predicted; Li et al. 2010)
***Equus caballus* (Horse - class *Mammalia*)**								
Uncharacterised Protein	F7BJ10	77	579	HTEL	horse-f7bj10	-	892 / 0.0	Hydrolase activity (evp: predicted; Wade et al. 2009)
Uncharacterised Protein (*CES5A*)	F7C7A8	48	513	ETLP	horse-f7c7a8	-	483 / 1e-136	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Wade et al. 2009)
Uncharacterised Protein	F6VXP7	46	565	HVEL	horse-f6vxp7	-	485 / 1e-137	Hydrolase activity (evp: predicted; Wade et al. 2009)
Uncharacterised Protein	F6ZMG7	46	567	HVEL	horse-f6zmg7	-	484 / 1e-137	Hydrolase activity (evp: predicted; Wade et al. 2009)
Uncharacterised Protein	F6UN85	46	565	HVEL	horse-f6un85	-	484 / 1e-137	Hydrolase activity (evp: predicted; Wade et al. 2009)
***Loxodonta africana* (African bush elephant - class *Mammalia*)**								
Ces2	G3TN98	76	554	HTEL	loxaf-g3tn98	-	852 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised Protein (*CES5A*)	G3T3N6	49	575	FFAP	loxaf-g3t3n6	-	487 / 1e-138	Hydrolase activity (evp: predicted)
Uncharacterised Protein	G3TEZ9	47	537	EHIE	loxaf-g3tez9	-	496 / 1e-140	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)

Uncharacterised Protein	G3T504 (*G3TND5*)	47	565	HIEL	loxaf-g3t504	-	493 / 1e-139	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
Uncharacterised Protein	G3UGH4	45	566	HIEL	loxaf-g3ugh4	-	475 / 1e-134	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
Uncharacterised Protein (*CES3*)	G3SP52	44	576	QEEL	loxaf-g3sp52	-	474 / 1e-134	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
***Canis lupus familiaris* (Dog - class *Mammalia*)**								
Ces2	F1P6W8	74	585	HTEL	canfa-f1p6w8	-	855 / 0.0	Hydrolase activity (evp: predicted; Lindblad-Toh et al. 2005)
Carboxylesterase 5A	Q6AW47 (*F1PQX8*)	48	575	SSAP	canfa-cauxin	NM_001003969, AB186392	493 / 1e-139	Hydrolase activity (evp: transcript level; Lindblad-Toh et al. 2005)
Carboxylesterase D1	F1PQV5 (*replaced by Q95N05*)	47	565	HVEL	canfa-CESDD1	AB023629; AAEX03001588	498 / 1e-141	Hydrolase activity (evp: transcript level; Lindblad-Toh et al. 2005)
***Oryctolagus cuniculus* (European rabbit - class *Mammalia*)**								
Uncharacterised Protein	G1TZV1	74	558	HTEL	rabit-g1tzv1	AAGW02053044.1	853 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised Protein	G1SJQ8	74	621	HTEL	rabit-g1sjq8	AAGW02067905.1	852 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised protein	G1T6X7	74	558	HTEL	rabit-g1t6x7	AAGW02053044.1	850 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised protein	G1TDR0	74	534	HTEL	rabit-g1tdr0	AAGW02067906.1	820 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised protein	G1T7P3	73	532	HTEL	rabit-g1t7p3	AAGW02053044.1	825 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised protein	G1T7Q5	73	561	HTEL	rabit-g1t7q5	AAGW02053044.1	813 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised protein	G1SN51	72	561	HTEL	rabit-g1sn51	AAGW02067906.1 AGW02067907.1	832 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised protein	G1TMC5	72	556	HTEL	rabit-g1tmc5	AAGW02053046.1	808 / 0.0	Hydrolase activity (evp: predicted)
Liver carboxylesterase 2	P14943	72	532	HTEL	rabit-2cxes	-	798 / 0.0	Detoxification of xenobiotics and activation of ester and amide prodrugs. (evp:protein level; Ozols J 1989)
Uncharacterised protein	G1SF18	72	422	HKEL	rabit-g1sf18	AAGW02067904	633 / 0.0	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
Uncharacterised protein	G1T6L1	71	528	HTEL	rabit-g1t6l1	AAGW02053044.1	778 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised protein	G1T441	71	470	FARN	rabit-g1t441	AAGW02053044	681 / 0.0	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)

Uncharacterised protein	G1T867	70	469	ARNG	rabit-g1t867	AAGW02053044	665 / 0.0	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
Uncharacterised protein	G1SF73	67	552	QTEL	rabit-g1sf73	-	739 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised protein (*Ces5A*)	G1SRM0	48	536	LSSL	rabit-g1srm0	AAGW02052800	493 / 1e-140	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
Uncharacterised protein	G1SRL5	45	564	HIEL	rabit-g1srl5	AAGW02052800	473 / 1e-133	Hydrolase activity (evp: predicted)
***Rattus norvegicus* (Brown rat - class *Mammalia*)**								
Ces2h	Q32Q55	73	558	HTEL	ratno-q32q55	BC107806.1	858 / 0.0	Carboxylesterase activity (evp: transcript level; Gibbs et al. 2004)
LOC679149 protein	Q4QR68	70	561	HTEL	ratno-q4qr68	BC097486.1	797 / 0.0	Carboxylesterase activity (evp: transcript level)
Ces2E	G3V7J5	70	557	HTEL	ratno-phebest	D50580.1, CH474006.1	792 / 0.0	Hydrolase activity (evp: predicted; Gibbs et al. 2004)
Ces2c	G3V9D8	70	561	HAEL	ratno-pbcxe	AB010635.1, CH473986.1	778 / 0.0	Hydrolase activity (evp: predicted; Gibbs et al. 2004)
Carboxylesterase (*Ces2*)	O70177	69	561	HAEL	ratno-sicxe	AB010632	789 / 0.0	Carboxylesterase activity (evp: predicted)
Ces2g	D3ZXQ0	67	560	HKEL	ratno-d3zxq0	CH473972.1	759 / 0.0	Carboxylesterase activity (evp: predicted; Gibbs et al. 2004)
Ces2j	D3ZP14	67	556	HAEL	ratno-d3zp14	-	726 / 0.0	Hydrolase activity (evp: predicted; Gibbs et al. 2004)
Carboxylesterase (Protein Ces2a)	Q8K3R0	66	558	HAEL	ratno-LOC246252	NM_144743, AY034877	757 / 0.0	Carboxylesterase activity (evp: transcript level; Gibbs et al. 2004)
Ces2i	D3ZE31	66	559	HAEL	ratno-d3ze31	-	742 / 0.0	Carboxylesterase activity (evp: predicted; Gibbs et al. 2004)
Carboxylesterase 1D	P16303 (*G3V822*)	46	565	HVEL	ratno-Ces3	X65296, AF171640, L81144, BC061789, X51974, X65296, L46791, X51974, CH474037	496 / 1e-140	Major lipase in white adipose tissue; carboxylesterase activity and long-chain-fatty-acyl ethyl ester hydrolase activity (evp: protein level; Robbi et al. 1990)
Carboxylesterase 1C	P10959	46	549	TEHT	ratno-est2	D30620, D00362, BC088251, M20629, X78489	480 / 1e-135	Carboxylesterase activity (evp: protein level; Takagi et al. 1998)
Carboxylesterase 1E	Q63108	46	561	HTEL	ratno-3cxes	AY387066	476 / 1e-134	Carboxylesterase activity (evp: transcript level)
Carboxylesterase-like	Q68G49 (*P80250*)	45	565	HVEL	ratno-q68g49	BC078681	483 / 1e-136	Hydrolase activity (evp: predicted; Gerhard et al. 2004). Note: P80250 is classified as Palmitoyl-CoA hydrolase (64 a.a.)

***Cavia porcellus* (Guinea pig - class *Mammalia*)**								
Uncharacterised protein (*CES2*)	H0V5V8	73	568	HTEL	cavpo-h0v5v8	-	844 / 0.0	Carboxylesterase activity (evp: predicted)
Uncharacterised protein (*CES5A*)	H0V479	48	542	NIVP	cavpo-h0v479	-	479 / 1e-135	Hydrolase activity (evp: predicted)
Uncharacterised protein	H0VJ36	46	561	RSRH	cavpo-h0vj36	-	484 / 1e-137	Carboxylesterase activity (evp: predicted)
Uncharacterised protein	H0V1B7	45	565	HVEL	cavpo-h0v1b7	-	480 / 1e-135	Carboxylesterase activity (evp: predicted)
Uncharacterised protein (*Ces1d*)	H0VHN0 (*H0VU94*)	45	565	HTEL	cavpo-cxest	D67037, AB010634	476 / 1e-134	Hydrolase activity (evp: predicted)
***Bos taurus* (Aurochs - class *Mammalia*)**								
Ces2 (intestine, liver)	Q3T0R6 (*F1MU22*)	72	553	HTEL	bovin-q3t0r6	BC102288.1, AAFC03046191.1	812 / 0.0	Carboxylesterase activity (evp: transcript level)
Uncharacterised protein (*CES5A*)	E1BN79	49	576	SFAP	bovin-e1bn79	AAFC03022775	483 / 1e-136	Hydrolase activity (evp: predicted; Zimin et al. 2009)
Carboxylesterase 1	Q2KJ30, (*Q0VCI3, F1MTP2*)	46	566	HVEL	bovin-q2kj30	BC105548, BC120153, AAFC03040943, AAFC03073989, AAFC03119755, AAFC03119756	491 / 1e-139	Carboxylesterase activity (evp: transcript level); Note, alternative form Q0VCI3 shows a different C-terminus a.a. - RHHT)
Retinyl ester hydrolase type 1	Q5MYB8 (*Q3SZM8*)	46	565	HVEL	bovin-q5myb8	AY369075, BC102781	483 / 1e-137	Carboxylesterase activity (evp: transcript level; Zimin et al. 2009)
***Heterocephalus glaber* (Naked mole rat - class *Mammalia*)**								
Carboxylesterase 2	G5BZE3	72	553	HAEL	hetga-g5bze3	JH172552.1	815 / 0.0	Hydrolase activity (evp: predicted; Kim et al. 2011)
Carboxylesterase 2	G5BDH8	71	523	LFLL	hetga-g5bdh8	JH169662	717 / 0.0	Hydrolase activity (evp: predicted; Kim et al. 2011)
Carboxylesterase 2	G5BP68	70	570	AAQE	hetga-g5bp68	JH171227.1	788 / 0.0	Hydrolase activity (evp: predicted; Kim et al. 2011)
Carboxylesterase 2	G5BP66	69	562	HAEL	hetga-g5bp66	JH171227.1	781 / 0.0	Hydrolase activity (evp: predicted; Kim et al. 2011)
Carboxylesterase 2	G5BP67	67	527	PDEL	hetga-g5bp67	JH171227	704 / 0.0	Hydrolase activity (evp: predicted; Kim et al. 2011)
Carboxylesterase 2	G5BP65	61	534	HTEL	hetga-g5bp65	JH171227	672 / 0.0	Hydrolase activity (evp: predicted; Kim et al. 2011)
Carboxylesterase 3	G5BSD4	47	553	HMEE	hetga-g5bsd4	JH171634	480 / 1e-135	Hydrolase activity (evp: predicted; Kim et al. 2011)
Carboxylesterase 3	G5AS32	46	566	HIEL	hetga-g5as32	JH166723	486 / 1e-137	Hydrolase activity (evp: predicted; Kim et al. 2011)

Liver Carboxylesterase 1	G5AMH8	45	582	HTEL	hetga-g5amh8	JH165998	482 / 1e-136	Hydrolase activity (evp: predicted; Kim et al. 2011)
***Mus musculus* (House mouse - class *Mammalia*)**								
Ces2h	F6Z9B9	72	558	HKEL	mouse-Ces2h	AC166833.4, XM_488149.1	850 / 0.0	Carboxylesterase activity (evp: predicted; Church et al. 2009)
Ces2c	Q91WG0	71	561	HREL	mouse-Ces2c	BC015290.1, AC166833.4	812 / 0.0	Carboxylesterase activity (evp: transcript level; Furihata et al. 2003)
Uncharacterised protein Ces2d-ps	D3YWM6	71	558	HREL	mouse-Ces2d-ps	-	799 / 0.0	Hydrolase activity (evp: predicted; Church et al. 2009)
Ces2b	Q6PDB7	71	556	HTEL	mouse-Ces2b	BC058815.1	783 / 0.0	Carboxylesterase activity (evp: transcript level; Mural et al. 2002)
Carboxylesterase 5 (Protein Ces2e)	Q8BK48	70	559	HKEL	mouse-Ces2e	XM_134366, BC022148	817 / 0.0	Carboxylesterase activity (evp: transcript level)
Ces2g	E9PV38	68	560	HKEL	mouse-Ces2g	BC027185.1, BC024548.1, BC026641.1	779 / 0.0	Carboxylesterase activity (evp: transcript level; Church et al. 2009)
Putative uncharacterised protein Ces2a Ces6	Q3TMR2 (*E9Q3D0*)	67	525	HAEL	mouse-Ces2a	BC024491.1, BC024517.1, BC025537.1	756 / 0.0	Carboxylesterase activity (evp: transcript level)
Ces2f	Q08ED5 (*Q149K3*)	64	561	IKAV	mouse-Ces2f	AC166833, BC117743	721 / 0.0	Carboxylesterase activity (evp: transcript level; Church et al. 2009)
Carboxylesterase 5A (*Ces5a, Ces7*)	Q6AW46	47	575	SAAS	mouse-cauxin	NM_001003951, XM_357913, AB186393, AC124591, AC162945, AK007235	479 / 1e-135	Hydrolase activity (evp: transcript level; Miyazaki et al. 2006)
Carboxylesterase 1D (Carboxylesterase 3)	Q8VCT4	46	565	HVEL	mouse-Ces1d	AF378751, AB025028, AB023631, AK078879, BC019198	486 / 1e-137	Carboxylesterase activity (evp: protein level; Furihata et al. 2004)
Carboxylesterase 1E	Q64176 (*Q3UN14, H3BL34*)	45	562	HTEL	mouse-Ces1e	S80191, AK144549, AC162949	483 / 1e-137	Carboxylesterase activity (evp: protein level; Ovnic et al. 1991)
***Cricetulus griseus* (Chinese hamster - class *Mammalia*)**								
Carboxylesterase 2	G3IIG3	71	511	HGEL	crigr-g3iig3	JH003006.1	783 / 0.0	Hydrolase activity (evp: predicted; Xu et al. 2011)
Liver carboxylesterase	G3IIG1	70	561	HKEL	crigr-g3iig1	JH003006.1	815 / 0.0	Hydrolase activity (evp: predicted; Xu et al. 2011)

Liver Carboxylesterase	G3I767	70	535	HKEL	crigr-g3i767.2	JH001411.1	780 / 0.0	Hydrolase activity (evp: predicted; Xu et al. 2011)
Liver carboxylesterase	G3I766	69	561	HQEL	crigr-g3i766	JH001411.1	801 / 0.0	Hydrolase activity (evp: predicted; Xu et al. 2011)
Liver carboxylesterase	G3I769	67	545	HAEL	crigr-g3i769	JH001411.1	742 / 0.0	Hydrolase activity (evp: predicted; Xu et al. 2011)
Liver carboxylesterase	G3IIG0	64	529	NKNV	crigr-g3iig0	JH003006	714 / 0.0	Hydrolase activity (evp: predicted; Xu et al. 2011)
Liver carboxylesterase	G3I770	61	420	HAEL	crigr-g3i770	JH001411	551 / 1e-157	Hydrolase activity (evp: predicted; Xu et al. 2011)
Liver carboxylesterase	G3I768	57	449	HTEL	crigr-g3i768	JH001411	546 / 1e-156	Hydrolase activity (evp: predicted; Xu et al. 2011)
Liver carboxylesterase 1	G3I7X9	46	765	HVEL	crigr-g3i7x9.2	JH001461	481 / 1e-136	Hydrolase activity (evp: predicted; Xu et al. 2011)
***Mesocricetus auratus* (Golden hamster - class *Mammalia*)**								
Carboxylesterase	O35533	70	559	HQEL	mesau-cxest2	D50577	807 / 0.0	Carboxylesterase activity (evp: trancript level; Sone et al. 1994)
Liver carboxylesterase	Q64419	66	561	HSEL	mesau-cxest	D28566.1	743 / 0.0	Detoxification of xenobiotics and activation of ester and amide prodrugs. (evp: transcript level; Sone et al. 1994)
Carboxylesterase	O35534	46	565	HAEL	mesau-cxest3	D50578	487 / 1e-138	Carboxylesterase activity (evp: trancript level; Sone et al. 1994)
***Otolemur garnettii* (Small-eared galago - class *Mammalia*)**								
Uncharacterised protein	H0WQ24	62	575	PEEP	otoga-h0wq24	AAQR03153135, AAQR03153136, AAQR03153137, AAQR03153138, AAQR03153139	670 / 0.0	Hydrolase activity (evp: predicted)
Uncharacterised protein	H0X743	46	566	HIEL	otoga-h0x743	AAQR03092733, AAQR03092734, AAQR03092735, AAQR03092736, AAQR03092737, AAQR03092738	489 / 1e-138	Hydrolase activity (evp: predicted)
***Sarcophilus harrisii* (Tasmanian devil - class *Mammalia*)**								
Uncharacterised protein	G3W1A9 (*G3W1B0*)	58	551	RVEL	sarha-g3w1a9	AEFK01033471, AEFK01033472	645 / 0.0	Hydrolase activity (evp: predicted; Miller et al. 2011)
Uncharacterised protein	G3VZX8	58	551	DQQS	sarha-g3vzx8	AEFK01033470, AEFK01033471	638 / 0.0	Hydrolase activity (evp: predicted; Miller et al. 2011)

Uncharacterised protein	G3VGT0 (*G3VGT1*)	49	568	RSEL	sarha-g3vgt0	AEFK01039751, AEFK01039752	496 / 1e-140	Hydrolase activity (evp: predicted; Miller et al. 2011)
Uncharacterised protein	G3VRX2	48	563	HIEL	sarha-g3vrx2	AEFK01037694	521 / 1e-148	Hydrolase activity (evp: predicted; Miller et al. 2011)
Uncharacterised protein	G3VRX3	48	567	HIEL	sarha-g3vrx3	AEFK01037694	520 / 1e-147	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Miller et al. 2011)
Uncharacterised protein	G3VVI8	48	532	HKRL	sarha-g3vvi8	AEFK01033463, AEFK01033464, AEFK01033465, AEFK01033466	489 / 1e-138	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Miller et al. 2011)
Uncharacterised protein	G3VIX0	46	565	ITEL	sarha-g3vix0	AEFK01041367, AEFK01041368, AEFK01041369, AEFK01041370	481 / 1e-136	Hydrolase activity (evp: predicted; Miller et al. 2011)
***Monodelphis domestica* (Gray short-tailed opossum - class *Mammalia*)**								
Uncharacterised protein	F7GEP4 (*F7C7S8*, *F7C7R4*)	58	535	RMEL	mondo-f7gep4	-	630 / 0.0	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Mikkelsen et al. 2007)
Carboxylesterase 2-like protein 1 (*Uncharacterised protein*)	B2BSF5 (*F7C7U9, 8A4, 882, 893, 875, 897, 888, 870, XF9*)	58	550	DHDY	mondo-b2bsf5	EU019537	625 / 1e-179	Hydrolase activity (evp: transcript level; Holmes et al. 2008). Note: F7C7U9 to XF9 described as fragments; evp: predicted.
Uncharacterised protein	F7C7T8	56	487	RMEL	mondo-f7c7t8	-	592 / 1e-169	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Mikkelsen et al. 2007)
Carboxylesterase 1	B2BSZ5 (*F7GJ65, 72, F7GJF1, GAF2, F6TPQ8, K2, H0, F6TNT0, Q6*)	49	509	NITL	mondo-b2bsz5	EU074630	481 / 1e-136	Hydrolase activity (evp: transcript level; note: described as fragment in UniProt; Holmes et al. 2008). Note: HIEL appears in the alternative fragments, in UniProt
Uncharacterised protein	F7C7P0	47	541	HVEL	mondo-f7c7p0	-	481 / 1e-136	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Mikkelsen et al. 2007)
Uncharacterised protein	F7G265	46	539	HVEL	mondo-f7g265	-	484 / 1e-137	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Mikkelsen et al. 2007)

Uncharacterised protein	F7C7R1	46	540	HVEL	mondo-f7c7r1	-	479 / 1e-135	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Mikkelsen et al. 2007)
Uncharacterised protein	F7C7P6	46	540	HVEL	mondo-f7c7p6	-	478 / 1e-135	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Mikkelsen et al. 2007)
Uncharacterised protein	F6SLK2	45	531	EKPV	mondo-f6slk2	-	475 / 1e-134	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Mikkelsen et al. 2007)
***Anolis carolinensis* (Green anole - class *Reptilia*)**								
Uncharacterised protein	H9GHC5	50	562	HQEL	anoca-h9ghc5	-	541 / 1e-154	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
Uncharacterised protein	H9GB75	49	571	HQEL	anoca-h9gb75	-	538 / 1e-153	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
Uncharacterised protein	H9G4C9	49	559	HREL	anoca-h9g4c9	-	531 / 1e-151	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
Uncharacterised protein	H9GGH4	48	564	HREL	anoca-h9ggh4	-	514 / 1e-146	Hydrolase activity (evp: predicted)
Uncharacterised protein	H9GCS3	48	546	DTPR	anoca-h9gcs3	-	501 / 1e-142	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
***Xenopus laevis* (African clawed frog - class *Amphibia*)**								
LOC443703 protein	Q6GM54	50	568	HVEL	xenla-q6gm54	BC074230	526 / 1e-149	Hydrolase activity (evp: transcript level; note: described as fragment in UniProt)
Putative uncharacterised protein	Q52L41, (*Q32N39, A1L2G7, Q0IH56*)	50	587	HVEL	xenla-q52l41	BC094077, BC108855, BC129520, BC123305	521 / 1e-148	Hydrolase activity (evp: transcript level; note: described as fragment in UniProt)
***Xenopus tropicalis* (Western frog - class *Amphibia*)**								
Uncharacterised protein	F6RDU1 (*F6YVF9*)	51	561	HVEL	xentr-LOC394897	BC064228, AAMC01050003, AAMC01050004, AAMC01050005, AAMC01050006, AAMC01050007	526 / 1e-149	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Hellsten et al. 2010)
Uncharacterised protein	F6Y4C8	50	575	HVEL	xentr-f6y4c8	AAMC01050011, AAMC01050012, AAMC01050013, AAMC01050014, AAMC01050015, AAMC01050016, AAMC01050017	525 / 1e-149	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Hellsten et al. 2010)

Putative uncharacterised protein MGC89138 (*Ces2*)	Q640T6 (*F6YVB0*)	50	557	RVQL	xentr-cxest2	BC082503, AAMC01050001, AAMC01050002	521 / 1e-148	Hydrolase activity (evp: transcript level; note: described as fragment in UniProt)
LOC100144981 protein (*Ces3*)	B0BM77 (*F7DR63*)	50	557	RIEL	xentr-b0bm77	BC158318, AAMC01050018, AAMC01050019, AAMC01050020	517 / 1e-147	Hydrolase activity (evp: transcript level; Hellsten et al. 2010)
Uncharacterised protein (*Ces3*)	F6YVE5	48	584	HIEL	xentr-f6yve5	AAMC01136814, AAMC01136815	512 / 1e-145	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Hellsten et al. 2010)
Uncharacterised protein	F6V2J6	48	564	RAEL	xentr-f6v2j6	AAMC01049999	494 / 1e-140	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Hellsten et al. 2010)
***Taeniopygia guttata* (Zebra finch - class *Aves*)**								
Uncharacterised protein	H0ZHA8	49	528	HTDL	taegu-h0zha8	-	498 / 1e-141	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Warren et al. 2010)
***Ornithorhynchus anatinus* (Duck-billed platypus - class *Mammalia*)**								
Uncharacterised protein	F6S0Q0 (*F7BGV4*)	47	564	RTEL	ornan-f6s0q0	-	506 / 1e-143	Hydrolase activity (evp: predicted; note: described as fragment in UniProt; Warren et al. 2008)
***Anas platyrhynchos* (Mallard – class *Aves*)**								
Fatty acyl-CoA hydrolase precursor, medium chain (thioesterase B)	Q04791	47	557	HTDL	anapl-thioe	L05493	497 / 1e-141	Hydrolase activity; fatty acid biosynthetic process (evp: protein level; Hwang et al. 1993)
***Myotis lucifugus* (Little brown bat - class *Mammalia*)**								
Uncharacterised protein (*CES5A*)	G1PJM4	47	576	SFSH	myolu-g1pjm4	AAPE02051406	485 / 1e-137	Hydrolase activity (evp: predicted)
Uncharacterised protein	G1Q4P0	47	538	RTHT	myolu-g1q4p0	AAPE02051407	483 / 1e-137	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
Uncharacterised protein	G1QAF3	46	566	RIEL	myolu-g1qaf3	AAPE02051406, AAPE02051407	490 / 1e-139	Hydrolase activity (evp: predicted; note: described as fragment in UniProt)
Uncharacterised protein	G1NW29 (*G1PHM4, G1Q3Y2*)	46	565	HIEL	myolu-g1nw29	AAPE02051407	488 / 1e-138	Hydrolase activity (evp: predicted)
***Sus scrofa* (Pig - class mammalia)**								
Uncharacterised protein	F1RF14	46	565	HAEL	sussc-O97582	AF064741, CU694664	488 / 1e-138	Hydrolase activity (evp: predicted). Note: UniProt sequence is incomplete; GeneBank sequence was used as reference.

Liver carboxylesterase	Q29550	46	566	HAEL	sussc-cxest	X63323, EF525540, CU694664	488 / 1e-138	Hydrolase activity (evp: protein level; David et al. 1998)
Alternative pig liver esterase	A9GYW6	46	548	HAEL	sussc-a9gyw6	AM774149	485 / 1e-137	Carboxylesterase activity (evp: transcript level; note: described as fragment in UniProt; Hermann et al. 2008)
***Felis catus* (Domestic cat - class *Mammalia*)**								
Carboxylesterase (*CES-K1*)	Q766D7	46	566	HVEL	felca-CESK1	AB114676	488 / 1e-138	Hydrolase activity (evp: transcript level; Miyazaki et al. 2006)
Carboxylesterase (*CES1*)	Q864S9	46	566	HVEL	felca-CES1	AB094147	483 / 1e-136	Hydrolase activity (evp: transcript level; Miyazaki et al. 2006)
***Gallus gallus* (Chicken – class *Aves*)**								
Uncharacterised protein (*CES1*)	E1BYN1 (*F1NYT3*)	48	557	RTDL	chick-e1byn1	AADN02031822, AADN02031823, AADN02031824, AADN02031825	484 / 1e-137	Hydrolase activity (evp: predicted; Hillier et al. 2004 for International Chicken Consortium).
***Macaca fascicularis* (Long-tailed macaque - class *Mammalia*)**								
Liver carboxylesterase 1	O46421	47	566	HIEL	macfa-cxest	AB010633	481 / 1e-136	Carboxylesterase activity (evp: trancript level)
***Microtus ochrogaster* (Prairie vole - class *Mammalia*)**								
Esterase 1	E0V882	47	547	HTEL	micoh-e0v882	DP001217	479 / 1e-135	Hydrolase activity (evp: predicted)
Esterase 1	E0V887	47	547	HTEL	micoh-e0v887	DP001218	479 / 1e-135	Hydrolase activity (evp: predicted)
***Salmo salar* (Atlantic salmon - class *Actinopterygii*)**								
Fatty acyl-CoA hydrolase, medium chain (*SASB*)	C0PUR6	45	556	SQGN	salsa-c0pur6	BT072689	474 / 1e-134	Hydrolase activity (evp: transcript level; note: described as fragment in UniProt)
***Brachydanio rerio* (Zebrafish - class *Actinopterygii*)**								
Novel protein similar to vertebrate carboxylesterase family (*Ces3*)	Q1LUZ9 (*Q5BJI3, E7FCK7, F1R2A2, F1R9X5*)	45	546	LHTA	danre-q5bji3	BC091470, BX908765	474 / 1e-134	Hydrolase activity (evp: transcript level)

A.2 Maps of plasmid vectors

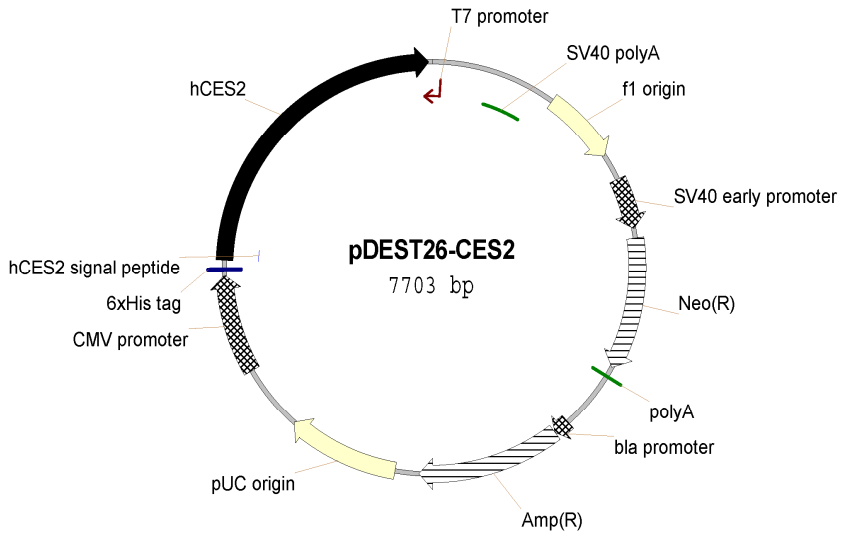

Figure A.1 pDEST26-CES2 vector map. *Generated with Vector NTI v.6.0. software (Invitrogen). Plasmid vector mentioned in Section 2.*

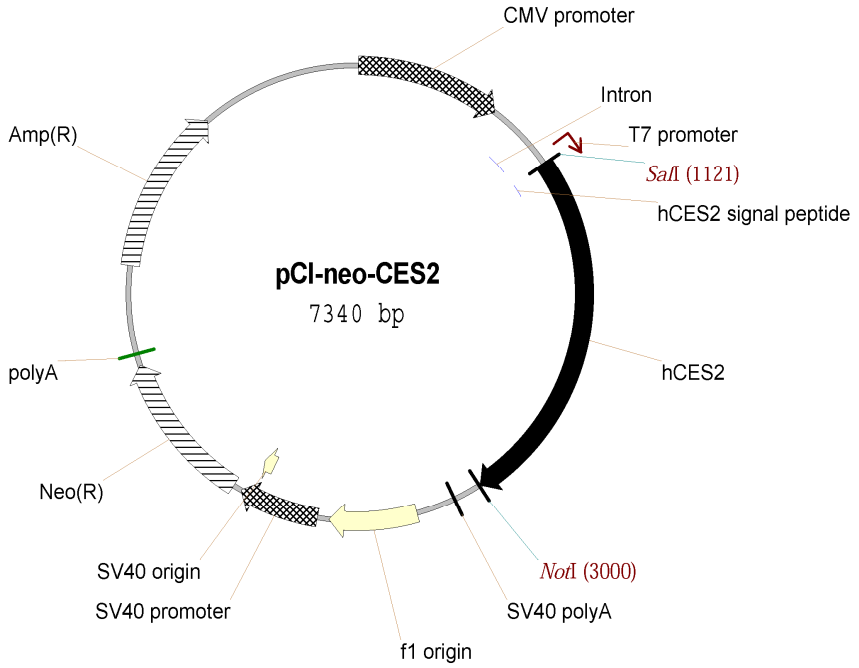

Figure A.2 pCI-neo-CES2 vector map. *Generated in Vector NTI v.6.0. software. Plasmid vector mentioned in Sections 3 and 4.*

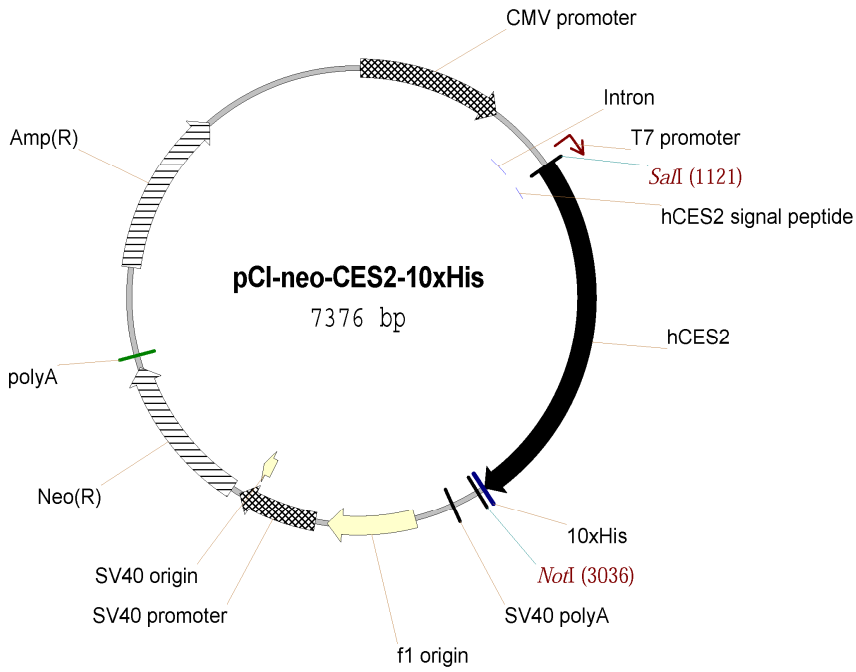

Figure A.3 pCI-neo-CES2-10xHis vector map. *Generated in Vector NTI v.6.0. software. Plasmid vector mentioned in Sections 3 and 4.*

Abbreviations: hCES2 – human carboxylesterase gene (geneID 8824); T7 – Escherichia coli bacteriophage T7 promoter; SV40 early promoter – eukaryotic simian virus 40 promoter and origin of replication enabling episomal replication in cell lines expressing SV-40 large T antigen; polyA – polyadenylation signal; Neo(R) – neomycin resistance gene; bla – ampicillin; Amp(R) – ampicillin resistance gene); pUC – prokaryotic replication origin (high copy); f1 – phage F1 replication origin (for ssDNA); CMV - cytomegalovirus; SalI and NotI restriction endonucleases; 6x or 10x His – tag of 6 or 10 histidines.

References

David L, Guo XJ, Moulin A, Piugserver A (1998) Purification and molecular cloning of porcine intestinal glycerol-ester hydrolase – evidence for its identity with carboxylesterase. *Eur J Biochem* 257: 142-148.

Furihata T, Hosokawa M, Koyano N, Nakamura T, Satoh T, Chiba K (2004) Identification of di-(2-ethylhexyl) phthalate-induced carboxylesterase 1 in C57BL/6 mouse liver microsomes: purification, cDNA cloning, and baculovirus-mediated expression. *Drug Metab Dispos* 32: 1170-1177.

Gerhard DS, Wagner L, Feingold EA, Shenmen CM, Grouse LH, Schuler G, Klein SL, Old S, Rasooly R, Good P, Guyer M, Peck AM, Derge JG, Lipman D, Collins FS, Jang W, Sherry S, Feolo M, Misquitta L, Lee E, Rotmistrovsky K, Greenhut SF, Schaefer CF, Buetow K, Bonner TI, Haussler D, Kent J, Kiekhaus M, Furey T, Brent M, Prange C, Schreiber K, Shapiro N, Bhat NK, Hopkins RF, Hsie F, Driscoll T, Soares MB, Casavant TL, Scheetz TE, Brown-stein MJ, Usdin TB, Toshiyuki S, Carninci P, Piao Y, Dudekula DB, Ko MS, Kawakami K, Suzuki Y, Sugano S, Gruber CE, Smith MR, Simmons B, Moore T, Waterman R, Johnson SL, Ruan Y, Wei CL, Mathavan S, Gunaratne PH, Wu J, Garcia AM, Hulyk SW, Fuh E, Yuan Y, Sneed A, Kowis C, Hodgson A, Muzny DM, McPherson J, Gibbs RA, Fahey J, Helton E, Ketteman M, Madan A, Rodrigues S, Sanchez A, Whiting M, Madari A, Young AC, Wetherby KD, Granite SJ, Kwong PN, Brinkley CP, Pearson RL, Bouffard GG, Blakesly RW, Green ED, Dickson MC, Rodriguez AC, Grimwood J, Schmutz J, Myers RM, Butterfield YS, Griffith M, Griffith OL, Krzywinski MI, Liao N, Morin R, Palmquist D, Petrescu AS, Skalska U, Smailus DE, Stott JM, Schnerch A, Schein JE, Jones SJ, Holt RA, Baross A, Marra MA, Clifton S, Makowski KA, Bosak S, Malek J; MGC Project Team (2004) The status, quality, and expansion of the NIH full-length cDNA project: the Mammalian Gene Collection (MGC). *Genom Res* 14: 2121-2127.

Hellsten U, Harland RM, Gilchrist MJ, Hendrix D, Jurka J, Kapitonov V, Ovcharenko I, Putnam NH, Shu S, Taher L, Blitz IL, Blumberg B, Dichmann DS, Dubchak I, Amaya E, Detter JC, Fletcher R, Gerhard DS, Goodstein D, Graves T, Grigoriev IV, Grimwood J, Kawashima T, Lindquist E, Lucas SM, Mead PE, Mitros T, Ogino H, Ohta Y, Poliakov AV, Pollet N, Robert J, Salamov A, Sater AK, Schmutz J, Terry A, Vize PD, Warren WC, Wells D, Wills A, Wilson RK, Zimmerman LB, Zorn AM, Grainger R, Grammer T, Khokha MK, Richardson PM, Rokhsar DS (2010) The genome of the Western clawed frog *Xenopus tropicalis*. *Science* 328: 633-636.

Hermann M, Kietzmann MU, Ivancić M, Zenzmaier C, Luiten RG, Skranc W, Wubbolts M, Winkler M, Birner-Gruenberger R, Pichler H, Schwab H (2008) Alternative pig liver esterase (APLE) - cloning, identification and functional expression in *Pichia pastoris* of a versatile new biocatalyst. *J Biotecnol* 133: 301-310.

Hwang CS, Kolattukudy PE (1993) Molecular cloning and sequencing of thioesterase B cDNA and stimulation of expression of the thioesterase B gene associated with hormonal induction of peroxisome proliferation. *J Biol Chem* 268: 14278-14284.

International Chicken Genome Sequencing Consortium (2004) Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature* 432: 695-716.

Miller W, Hayes VM, Ratan A, Petersen DC, Wittekindt NE; Miller J, Walenz B, Knight J, Qi J, Zhao F, Wang Q, Bedoya-Reina OC, Katiyar N, Tomsho LP, Kasson LM, Hardie RA, Woodbridge P, Tindall EA,

Bertelsen MF, Dixon D, Pyecroft S, Helgen KM, Lesk AM, Pringle TH, Patterson N, Zhang Y, Kreiss A, Woods GM, Jones ME, Schuster SC (2011) Genetic diversity and population structure of the endangered marsupial *Sarcophilus harrisii* (Tasmanian devil). *Proc Natl Acad Sci* 108: 12348-12353.

Mikkelsen TS, Wakefield MJ, Aken B, Amemiya CT, Chang JL, Duke S, Garber M, Gentles AJ, Goodstadt L, Heger A, Jurka J, Kamal M, Mauceli E, Searle SM, Sharpe T, Baker ML, Batzer MA, Benos PV, Belov K, Clamp M, Cook A, Cuff J, Das R, Davidow L, Deakin JE, Fazzari MJ, Glass JL, Grabherr M, Greally JM, Gu W, Hore TA, Huttley GA, Kleber M, Jirtle RL, Koina E, Lee JT, Mahony S, Marra MA, Miller RD, Nicholls RD, Oda M, Papenfuss AT, Parra ZE, Pollock DD, Ray DA, Schein JE, Speed TP, Thompson K, VandeBerg JL, Wade CM, Walker JA, Waters PD, Webber C, Weidman JR, Xie X, Zody MC; Broad Institute Genome Sequencing Platform; Broad Institute Whole Genome Assembly Team, Graves JA, Ponting CP, Breen M, Samollow PB, Lander ES, Lindblad-Toh K (2007) Genome of the marsupial *Monodelphis domestica* reveals innovation in non-coding sequences. *Nature* 447: 167-177.

Miyazaki M, Yamashita T, Hosokawa M, Taira H, Suzuki A (2006) Species-, sex-, and age-dependent urinary excretion of cauxin, a mammalian carboxylesterase. *Comp Biochem Physiol B Biochem Mol Biol* 145: 270-277.

Ovnic M, Swank RT, Fletcher C, Zhen L, Novak EK, Baumann H, Heintz N, Ganschow RE (1991) Characterization and functional expression of a cDNA encoding egasyn (esterase-22): the endoplasmic reticulum-targeting protein of beta-glucuronidase. *Genomics* 11: 956-967.

Robbi M, Beaufay H, Octave JN (1990) Nucleotide sequence of cDNA coding for rat liver pI 6.1 esterase (ES-10), a carboxylesterase located in the lumen of the endoplasmic reticulum. *Biochem J* 269: 451-458.

Sanghani SP, Quinney SK, Fredenburg TB, Davis WI, Murry DJ, Bosron WF (2004) Hydrolysis of irinotecan and its oxidative metabolites, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin and 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin, by human carboxylesterases CES1A1, CES2, and a newly expressed carboxylesterase isoenzyme, CES3. *Drug Metab Dispos* 32: 505-11.

Takagi Y, Morohashi K, Kawabata S, Go M, Omura T (1998) Molecular cloning and nucleotide sequence of cDNA of microsomal carboxyesterase E1 of rat liver. *J Biochem* 104: 801-806.

Warren WC, Clayton DF, Ellegren H, Arnold AP, Hillier LW, Künstner A, Searle S, White S, Vilella AJ, Fairley S, Heger A, Kong L, Ponting CP, Jarvis ED, Mello CV, Minx P, Lovell P, Velho TA, Ferris M, Balakrishnan CN, Sinha S, Blatti C, London SE, Li Y, Lin YC, George J, Sweedler J, Southey B, Gunaratne P, Watson M, Nam K, Backström N, Smeds L, Nabholz B, Itoh Y, Whitney O, Pfenning AR, Howard J, Völker M, Skinner BM, Griffin DK, Ye L, McLaren WM, Flicek P, Quesada V, Velasco G, Lopez-Otin C, Puente XS, Olender T, Lancet D, Smit AF, Hubley R, Konkel MK, Walker JA, Batzer MA, Gu W, Pollock DD, Chen L, Cheng Z, Eichler EE, Stapley J, Slate J, Ekblom R, Birkhead T, Burke T, Burt D, Scharff C, Adam I, Richard H, Sultan M, Soldatov A, Lehrach H, Edwards SV, Yang SP, Li X, Graves T, Fulton L, Nelson J, Chinwalla A, Hou S, Mardis ER, Wilson RK (2010) The genome of a songbird. *Nature* 464: 757-762.

Warren WC, Hillier LW, Marshall Graves JA, Birney E, Ponting CP, Grützner F, Belov K, Miller W, Clarke L, Chinwalla AT, Yang SP, Heger A, Locke DP, Miethke P, Waters PD, Veyrunes F, Fulton L, Fulton B,

Graves T, Wallis J, Puente XS, López-Otín C, Ordóñez GR, Eichler EE, Chen L, Cheng Z, Deakin JE, Alsop A, Thompson K, Kirby P, Papenfuss AT, Wakefield MJ, Olender T, Lancet D, Huttley GA, Smit AF, Pask A, Temple-Smith P, Batzer MA, Walker JA, Konkel MK, Harris RS, Whittington CM, Wong ES, Gemmell NJ, Buschiazzo E, Vargas Jentzsch IM, Merkel A, Schmitz J, Zemann A, Churakov G, Kriegs JO, Brosius J, Murchison EP, Sachidanandam R, Smith C, Hannon GJ, Tsend-Ayush E, McMillan D, Attenborough R, Rens W, Ferguson-Smith M, Lefèvre CM, Sharp JA, Nicholas KR, Ray DA, Kube M, Reinhardt R, Pringle TH, Taylor J, Jones RC, Nixon B, Dacheux JL, Niwa H, Sekita Y, Huang X, Stark A, Kheradpour P, Kellis M, Flicek P, Chen Y, Webber C, Hardison R, Nelson J, Hallsworth-Pepin K, Delehaunty K, Markovic C, Minx P, Feng Y, Kremitzki C, Mitreva M, Glasscock J, Wylie T, Wohldmann P, Thiru P, Nhan MN, Pohl CS, Smith SM, Hou S, Nefedov M, de Jong PJ, Renfree MB, Mardis ER, Wilson RK (2008) Genome analysis of the platypus reveals unique signatures of evolution. *Nature* 453: 175-183.

Zimin AV, Delcher AL, Florea L, Kelley DR, Schatz MC, Puiu D, Hanrahan F, Pertea G, Van Tassell CP, Sonstegard TS, Marçais G, Roberts M, Subramanian P, Yorke JA, Salzberg SL (2009) A whole-genome assembly of the domestic cow, *Bos taurus*. *Genome Biol* 10: R42.

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"A great pleasure in life is doing what people say you cannot do."

Walter Bagehot (1826-1877)

Thesis Cover: Composite image of a "laughing" Caco-2 clone, growing for 14 days without dividing, and the flow diagram developed for CES2 specific activity identification and quantification.